

FORM PTO-390 (Modified)  
(REV 11-2000)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

## TRANSMITTAL LETTER TO THE UNITED STATES

235.00210101

DESIGNATED/ELECTED OFFICE (DO/EO/US)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

CONCERNING A FILING UNDER 35 U.S.C. 371

10/030330

INTERNATIONAL APPLICATION NO.  
PCT/US00/10574INTERNATIONAL FILING DATE  
20 April 2000PRIORITY DATE CLAIMED  
21 April 1999

## TITLE OF INVENTION

A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN

## APPLICANT(S) FOR DO/EO/US

James TRAVIS, Jan S. POTEPA, and Daniel C. NELSON

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
  - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ has been communicated by the International Bureau.
  - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
- ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
  - a. ☐ is attached hereto.
  - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
- ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
  - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ have been communicated by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

## Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Formal Drawings; Paper version of Sequence Listing and Communication re. Sequence Listing; Election and Power of Attorney; itemized return postcard.

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.101) <b>10/030330</b>		INTERNATIONAL APPLICATION NO. <b>PCT/US00/10574</b>		ATTORNEY'S DOCKET NUMBER <b>235.00210101</b>	
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24. The following fees are submitted: <b>BASIC NATIONAL FEE ( 37 CFR 1.492 (a) (1) - (5) ) :</b>				<b>CALCULATIONS PTO USE ONLY</b>	
<input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... <b>\$1040.00</b>					
<input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... <b>\$890.00</b>					
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... <b>\$740.00</b>					
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... <b>\$710.00</b>					
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<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				<b>\$890.00</b>	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).				<b>\$130.00</b>	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	30 - 20 =	10	x \$18.00	<b>\$180.00</b>	
Independent claims	13 - 3 =	10	x \$84.00	<b>\$840.00</b>	
Multiple Dependent Claims (check if applicable). <input type="checkbox"/>				<b>\$0.00</b>	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				<b>\$2,040.00</b>	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				<b>\$1,020.00</b>	
<b>SUBTOTAL =</b>				<b>\$1,020.00</b>	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).				<b>\$0.00</b>	
<b>TOTAL NATIONAL FEE =</b>				<b>\$1,020.00</b>	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). <input type="checkbox"/>				<b>\$0.00</b>	
<b>TOTAL FEES ENCLOSED =</b>				<b>\$1,020.00</b>	
				Amount to be refunded	\$
				charged	\$

a. ☒ A check in the amount of **\$1,020.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. \_\_\_\_\_ in the amount of \_\_\_\_\_ to cover the above fees. A duplicate copy of this sheet is enclosed.


c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **13-4895**. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

David L. Provence  
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 SIGNATURE  
 David L. Provence  
 NAME  
 43,022  
 REGISTRATION NUMBER  
 October 19, 2001  
 DATE

Applicant(s): James Travis, et al.

Docket No.: 235.00210101

JC10 Rec'd PCT/PTO 19 OCT 2001

Title: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN

**BOX PCT**Assistant Commissioner for Patents  
Washington, D.C. 20231

We are transmitting the following documents along with this Transmittal Sheet (which is submitted in triplicate):

- ☒ Transmittal Letter to the United States DO/EO/US Concerning a Filing Under 35 U.S.C. 371 (Form PTO-1390, 2 signed copies, 2 pgs each).
- ☒ Formal Drawings (5 Figs on 7 sheets).
- ☐ A signed Declaration ( \_ pgs).
- ☒ Application Data Sheet ( 5 pgs).
- ☒ A signed Election Under 37 CFR 3.71, Revocation, Power of Attorney, and Certificate Under 3.73(b) (5 pgs, including copy of executed Assignment).
- ☐ An Assignment of the invention to \_ and Recordation Form Cover Sheet.
- ☐ A check in the amount of \$40.00 to pay the Assignment Recording Fee.
- ☒ A Preliminary Amendment (2 pgs).
- ☒ Computer readable form of Sequence Listing (1 diskette), paper copy of Sequence Listing (13 pgs), and Communication re. Sequence Listing (1 pg).
- ☒ Other: Copy of International Preliminary Examination Report; Copy of International Search Report.
- ☒ A check in the amount of \$1,020.00 to pay the filing fee.
- ☒ An itemized return postcard.

☒ Small Entity Status is entitled to be asserted in the above-identified application.

Please consider this a PETITION FOR EXTENSION OF TIME for a sufficient number of months to enter these papers and please charge any additional fees or credit overpayment to Deposit Account No. 13-4895. Triplicate copies of this sheet are enclosed.

MUEITING, RAASCH &amp; GEBHARDT, P.A.

Customer Number: 26813

By: David L. Provence

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**CERTIFICATE UNDER 37 CFR §1.10:**"Express Mail" mailing label number: EL 888271957 USDate of Deposit: October 19, 2001

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR §1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, BOX PCT, Washington, D.C. 20231.

By: Gara L. Ladwig

Name: Gara L. Ladwig

(SMALL ENTITY TRANSMITTAL UNDER RULE 1.10)

PATENT  
Attorney Docket No. 235.00210101

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): James Travis, et al. )  
Serial No.: Unknown )  
Filed: Even date herewith )  
For: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN )

PRELIMINARY AMENDMENT

**BOX PCT**

Assistant Commissioner for Patents  
Washington, D.C. 20231

Prior to examination and consideration of the above-identified U.S. national phase patent application, Applicants respectfully request entry of the following amendment to the application:

IN THE SPECIFICATION

Please insert the following new paragraph at page 1, line 5:

-- The present application is a national stage filing of International Patent Application No. PCT/US00/10574, filed on April 20, 2000, which in turn claims priority to U.S. Provisional Application Serial No. 60/130,436, filed April 21, 1999, both of which are incorporated herein by reference. --

**Preliminary Amendment**

Page 2

Inventors : James Travis, et al.

Attorney Docket No.: 235.00210101

Title: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN

**REMARKS**

This preliminary amendment adds a statement regarding related applications that makes reference to the U.S. and international priority applications. No new matter is added by way of this amendment.

If the Examiner wishes to discuss any issues concerning this application by telephone, please contact the below-signed attorney.

Respectfully submitted,  
JAMES TRAVIS, ET AL.  
By their Representatives,  
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October 19, 2001

Date  
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By:

David L. Provence

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Date of Deposit: 19 October 2001  
Express Mail label number: EL 888271957 US

I hereby certify that this paper and/or fee is/are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to "BOX PCT, Assistant Commissioner for Patents, Washington, D. C. 20231."

Name: Gara L. Ladwig

A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN

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Statement of Government Rights

The present invention was made with support from the National Institutes of Health under Grant No. DE09761. The government may have certain rights in the invention.

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Background

The anaerobe *Porphyromonas gingivalis* (*P. gingivalis*) has been implicated as a major causative organism of adult onset periodontal disease. Enzymes from this organism have been found to degrade several proteins, including, for example, collagen, fibrinogen, immunoglobulins, complement proteins, and fibronectin. Recent evidence has shown that three proteinases released from *P. gingivalis* may have a physiological role in modulating the human immune system in addition to their general ability to degrade proteins. These three proteinases are referred to as gingipains and include arginine-specific gingipain A (RgpA) and arginine-specific gingipain B (Rgp B), which are capable of specifically cleaving after arginine residues, and lysine-specific gingipain (Kgp) which is able to specifically cleave after lysine residues. Working in concert, these proteinases have been shown to produce bradykinin from high molecular weight kininogen, either directly or indirectly (kallikrein activation), resulting in the enhancement of vascular permeability (Imamura, et al., Infect. Immun., 63(5):1999-2003 (1995)). This mechanism, which is used to provide nutritional components for the growth and proliferation of *P. gingivalis*, is presumed to be responsible for both the increased gingival crevicular fluid (GCF) and edema clinically noted in periodontal pockets of patients with advanced periodontitis (Darany et al., J. Periodontol., 63:743-747 (1992)).

The interaction of *P. gingivalis* with a host's immune system response has been paradoxical, in that *P. gingivalis* has demonstrated both pro-

inflammatory and anti-inflammatory responses. For example, *P. gingivalis* lipopolysaccharide has been shown to increase mRNA levels of interleukin-8 (IL-8) in neutrophils (Sugita et al., Inflammation, 22(3):253-267(1998)), and gingipains R have been shown to increase neutrophil chemotaxis by release of

5 C5a from C5 of the complement system (Wingrove et al., J. Biol. Chem., 267(26):18902-18907 (1992)). However, these proteinases are also capable of cleaving the C5a receptor from infiltrating neutrophils (Jagels et al., Infect. Immun., 64(6):1984-1991 (1996)), thereby effectively neutralizing localized chemotactic activity. Additionally, *P. gingivalis* has the ability to inhibit both

10 IL-8 accumulation in gingival epithelial cells (Darveau et al., Infect. Immun., 66(4):1660-1665 (1998)), as well as transepithelial migration (Madianos et al., Infect. Immun., 65(10):3983-3990 (1997)).

These apparent activity contradictions may potentially be explained by the compartmentalization of the oral cavity, wherein distal activation of

15 chemotactic components and proximal paralysis of these factors creates a "leukocyte wall" between the periodontal plaque and gingival epithelium (Miyasaki, J. Periodontol., 62:761-774 (1991)). Indeed, it has been reported that soluble gingipains can stimulate IL-8 activity, whereas membrane bound gingipains, with a limited ability to diffuse beyond the plaque surface,

20 completely degrade IL-8 (Mikolajczyk-Pawlinska et al., FEBS Lett., 440:282-286 (1998)).

The recruitment of neutrophils to the "leukocyte wall" through both the increased leakage of blood vessels and a chemotactic gradient at first appear to be suicidal to *P. gingivalis*. However, such a scenario is not likely, as *P.*

25 *gingivalis* is known to have evolved mechanisms to survive in the presence of neutrophils. For example, *P. gingivalis* proteinases have been shown to degrade C3 complement and immunoglobulins (Schenkein et al., J. Immunol., 154:5331-5337 (1995)), thereby averting opsonization and subsequent detection by a host. Furthermore, gingipain R has been shown to have an inhibitory effect on the

30 oxidative burst utilized by neutrophils to kill microorganisms (Kadowaki et al., J. Biol. Chem., 269(33):21371-21378 (1994)). Similarly, the bacterial outer membrane of *P. gingivalis* may function as an antioxidant sink due to the

incorporation of large amounts of heme (Smalley et al., *J. Biochem.*, 331:681-685 (1998)).

Activated neutrophils in the leukocyte wall typically undergo degranulation due to the inability to phagocytize foreign organisms, thereby expelling large quantities of the proteinases human neutrophil elastase (HNE) and cathepsin G. Although these proteinases may cause abnormal connective tissue destruction, the presence of human plasma proteinase inhibitors (serpins) typically minimize connective tissue destruction by complexing with endogenous proteinases. These complexes are ultimately absorbed by the liver for degradation. For example, high protein levels of the  $\alpha_1$ -proteinase inhibitor ( $\alpha_1$ -PI) have been detected in GCF samples from patients diagnosed with severe periodontal disease (Huynh et al., *J. Clin. Periodontol.*, 19:187-192 (1992)). However, despite the presence of  $\alpha_1$ -PI, a high HNE activity is observed indicating that the  $\alpha_1$ -PI must be present in either complexed, oxidized, or proteolytically inactivated forms (Uitto et al., *J. Clin. Periodontol.*, 23:30-37 (1996)). This observation is supported by evidence showing that less than 35% of available  $\alpha_1$ -PI in the GCF is active as an inhibitor (Smith et al., *Archs. Oral Biol.*, 39(4):301-306 (1994)). Additionally, it has been shown that patients with  $\alpha_1$ -PI deficiencies have a significantly higher frequency of periodontal pocket depths  $\geq 5$  mm, thereby being predisposed to manifestations of periodontal disease (Fokkema et al., *J. Clin. Periodontol.*, 25:617-623 (1998)). Thus, there is a need for further understanding the interaction of such serpins with other oral bacterial proteinases.

## **Summary of the Invention**

Described herein are the isolation, purification and characterization of a polypeptide, particularly an oral bacterial polypeptide that interacts with a serpin such as the human serpin  $\alpha_1$ -PI. This polypeptide is referred to as "periodontain," not only because of its function as a proteinase, but also because this polypeptide may function as a putative factor in the dysregulation of serpin function in the periodontal cavity of an animal. Additionally, the deduced amino



acid sequence of periodontain, as determined by both partial peptide sequencing of the purified polypeptide and characterization of the *P. gingivalis* genome is provided.

Accordingly, the present invention provides an isolated oral bacterial polypeptide which has amidolytic activity for cleavage of a nondenatured human  $\alpha_1$ -proteinase inhibitor at a reactive site loop region of the inhibitor. The isolated polypeptide demonstrates amidolytic activity in a solution containing about 1 mM to about 500 mM Tris, about 500  $\mu$ M to about 100 mM cysteine maintained at a pH of about 7 to about 8. Preferably, the polypeptide is isolated from *Porphyromonas gingivalis* and is a cysteine proteinase.

The polypeptide of the invention preferably has a molecular weight of about 70 kD to about 80 kD as determined by gel filtration. The polypeptide of the invention will preferably cleave the reactive site loop region of the inhibitor represented by SEQ ID NO: 4 between glutamine and alanine and also between phenylalanine and leucine.

The present invention also provides an isolated polypeptide that is an oral bacterial cysteine proteinase and has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of the serpin. The isolated polypeptide is preferably isolated from *Porphyromonas gingivalis*. The isolated polypeptide of the invention further has the capability to cleave a target polypeptide nonspecifically.

The present invention further provides an isolated polypeptide that is isolated from *Porphyromonas gingivalis* and has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of the serpin.

The isolated polypeptide preferably contains an amino acid sequence having a percentage amino acid identity of greater than 37% to that of amino acid 148 to amino acid 843 of SEQ ID NO: 1. More preferably, the isolated polypeptide contains an amino acid sequence having a percentage amino acid identity of greater than 52% to that of amino acid 148 to amino acid 629 of SEQ ID NO: 1. In particularly preferred embodiments, the isolated polypeptide has an amino acid sequence represented by SEQ ID NO: 1, an active analog or an active fragment thereof, and more preferably, an amino acid sequence

represented by amino acid 148 to amino acid 843 of SEQ ID NO: 1, an active analog or an active fragment thereof.

The present invention further provides an isolated nucleic acid encoding an oral bacterial polypeptide which has amidolytic activity for cleavage of a nondenatured human  $\alpha_1$ -proteinase inhibitor at a reactive site loop region of the inhibitor. Also provided is an isolated nucleic acid encoding a polypeptide which is an oral bacterial cysteine proteinase and has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of the serpin. The present invention further provides an isolated nucleic acid encoding a polypeptide which is isolated from *Porphyromonas gingivalis* and has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of the serpin.

The isolated nucleic acid fragment of the invention preferably has a nucleotide sequence represented by SEQ ID NO: 2. The isolated nucleic acid fragment of the invention further preferably encodes a polypeptide having an amino acid sequence with a percentage amino acid identity of greater than 37% when compared to amino acid 148 to amino acid 843 of SEQ ID NO: 1.

The present invention also provides an isolated nucleic acid fragment as described herein wherein the complement of the nucleic acid fragment hybridizes to SEQ ID NO: 2 under hybridization conditions of 0.5 M phosphate buffer, pH 7.2, 7% sodium dodecyl sulfate (SDS), 10 mM ethylenediaminetetraacetate (EDTA), at 68°C, followed by three 20 minute washes in 2x SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS, at 65°C, wherein at least about 15 nucleotides of the complement hybridize.

The present invention further provides a method for identifying an inhibitor of a polypeptide which has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of a serpin. The method includes isolating an agent that inhibits the amidolytic activity of the polypeptide by incubating the polypeptide with the agent under conditions that promote amidolytic activity of the polypeptide and determining if the amidolytic activity of the polypeptide is reduced relative to the amidolytic activity of the

polypeptide in the absence of the agent. Preferably, the polypeptide is isolated form *Porphyromonas gingivalis*.

The invention also provides an immunogenic composition having a polypeptide which has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of a serpin and is capable of eliciting antibodies in an animal.

Also provided is a composition containing an inhibitor to a polypeptide that has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of a serpin isolated from an oral bacterium.

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### Definitions

"Polypeptide" as used herein refers to a polymer of amino acids and does not refer to a specific length of a polymer of amino acids. Thus, for example, the terms peptide, oligopeptide, protein, and enzyme are included within the definition of polypeptide. This term also includes post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations, and the like.

"Proteinase," "peptidase," and "protease" all refer to enzymes that catalyze the hydrolysis of peptide bonds in a polypeptide. A "peptide bond" or "amide bond" is a covalent bond between the alpha-amino group of one amino acid and the alpha-carboxyl group of another amino acid.

As used herein, the term "isolated" means that a polypeptide is either removed from its natural environment or synthetically derived. Preferably, the polypeptide is purified, i.e., essentially free from any other polypeptides and associated cellular products or other impurities.

"Amidolytic activity" refers to the ability of a polypeptide to catalyze the hydrolysis of at least one peptide bond in another polypeptide. The term "cleavage" can also be used to refer to the hydrolysis of a peptide bond in a polypeptide.

"Periodontain" refers to a polypeptide having amidolytic activity with respect to a target polypeptide. Preferably, the polypeptide is a cysteine proteinase that is isolated from an oral bacteria, such as, *P. gingivalis*. A "target

polypeptide," as used herein, includes any denatured polypeptide and/or a native polypeptide, i.e., nondenatured, that contains at least one exposed contiguous amino acid region, such as a random coil as determined for example, by x-ray crystallography, that is susceptible to cleavage, preferably by periodontain. The exposed amino acid region typically contains at least about 3 contiguous amino acids, and preferably at least about 10 contiguous amino acids. Periodontain target polypeptides include, for example, serpin polypeptides in general, and mammalian serpin polypeptides specifically. A preferred target polypeptide of the invention is the human serpin,  $\alpha_1$ -proteinase inhibitor ( $\alpha_1$ -PI) (SEQ ID NO: 3). With respect to a serpin polypeptide, the exposed amino acid region, e.g., random coil, as used herein, is referred to as a "reactive site loop" region or "RSL." The RSL region of human  $\alpha_1$ -PI is about 15 contiguous amino acids (SEQ ID NO: 4).

An "active analog" or "active fragment" of a polypeptide of the invention is one that has amidolytic activity by hydrolysis of a peptide bond present in the target polypeptide as described herein. Active analogs and active fragments are described in greater detail herein.

"Nucleic acid fragment" as used herein refers to a linear polymeric form of nucleotides of any length, either ribonucleotides or deoxynucleotides, and includes both double- and single-stranded DNA and RNA. A nucleic acid fragment may include both coding and noncoding regions that can be obtained directly from a natural source (e.g., a microorganism), or can be prepared with the aid of recombinant or synthetic techniques. A nucleic acid molecule may be equivalent to this nucleic acid fragment or it can include this fragment in addition to one or more other nucleotides or polynucleotides. For example, the nucleic acid molecule of the invention can be a vector, such as an expression or cloning vector.

"Percentage amino acid identity" refers to a comparison of the amino acids of two polypeptides as described herein. Amino acid alignment may be determined, for example, using the sequence alignment program CLUSTAL W available at [www.genome.ad.jp/SIT/CLUSTALW.html](http://www.genome.ad.jp/SIT/CLUSTALW.html). and percent amino acid

identity may be determined by BLAST 2 SEQUENCES at National Center for Biotechnology Information (NCBI) website: [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov).

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### **Brief Description of the Drawings**

#### **Figure 1. Sequence alignment of periodontain, *PrtT*, and streptopain.**

The aligned putative gene products of *prtT* (SEQ ID NO: 5) and periodontain (SEQ ID NO: 1), both deduced from *P. gingivalis* strain W83 genome, and streptopain (SEQ ID NO: 6), from *S. pyogenes*, were aligned according to multiple sequence alignment using the sequence alignment program CLUSTAL W and the percent amino acid identity was determined by BLAST 2 SEQUENCES at NCBI using the default matrix. The black box(es) indicate that the same amino acid is present in a compared sequence as indicated, and grey box(es) indicate that a similar amino acid, e.g., a conservative amino acid substitution, is present in a compared sequence as indicated. The putative catalytic cysteine and histidine residues of streptopain are marked (\*). The underlined residues indicate the obtained N-terminal sequence of the light chain of periodontain. The double underlined residues indicate the sequence obtained from both N-terminal sequencing of the heavy chain and DNA sequencing of the 69 basepair PCR product. Arrows indicate the putative cleavage sites necessary to form a mature periodontain heterodimer polypeptide from a nascent polypeptide.

#### **Figure 2. Proposed processing for the maturation of periodontain.**

Structure of the periodontain polypeptide with proposed processing sites based on N-terminal sequencing of the purified heterodimer.

**Figure 3. Distribution of periodontain activity in various *P. gingivalis* strains.**

Periodontain activity from the indicated strains measured against human  $\alpha_1$ -PI using the assay as described in Material and Methods. Cell associated activities (clear bar), vesicle associated activities (hatched bar), and soluble activities (solid bar) were normalized to 100% for each strain.

**Figure 4. The human  $\alpha_1$ -proteinase inhibitor amino acid sequence.**

The amino acid sequence of human  $\alpha_1$ -PI is shown. The RSL region of human  $\alpha_1$ -PI is identified.

**Figure 5. The nucleic acid sequence of periodontain.**

The nucleic acid sequence for the full-length nascent polypeptide of the invention is shown.

**Figure 6. The RSL region of human  $\alpha_1$ -PI.**

**Detailed Description of Preferred Embodiments**

Periodontal disease in an animal host, is typically characterized by inflammation of the periodontium manifested by recruitment of neutrophils which can degranulate, releasing powerful proteinases responsible for destruction of connective tissues, and eventual loss of tooth attachment in the animal host. Although the presence of a host's innate proteinase inhibitors (i.e., serpins) may minimize tissue damage by endogenous proteinases, this is not clinically observed, and it has been speculated that proteolytic inactivation of a host's proteinase inhibitors may contribute to progression of the disease. A major pathogen associated with periodontal disease in an animal is the gram negative oral bacteria, *Porphyromonas gingivalis*. "Animal," as used herein, includes, for example, mammals, such as humans, sheep and dogs.

Described herein is a preferred polypeptide that has been isolated from the culture supernatants of *Porphyromonas gingivalis*. The polypeptide is capable of inactivating, for example, the nondenatured human serpin  $\alpha_1$ -

proteinase inhibitor ( $\alpha_1$ -PI), which is the primary endogenous regulator of human neutrophil elastase (HNE). A preferred polypeptide, referred to herein as periodontain, belongs to the cysteine proteinase family, and as a mature polypeptide, exists as a heterodimer. Periodontain (SEQ ID NO: 1) shares  
5 limited amino acid sequence identity to streptopain (SEQ ID NO: 6), a proteinase from *Streptococcus pyogenes*, and *priT* (SEQ ID NO: 5), a putative proteinase from *P. gingivalis* (Table III and Figure 1). Thus, a preferred polypeptide of the invention typically has a percentage amino acid identity of greater than 37% of the amino acid sequence including amino acid 148 to amino  
10 acid 843 of SEQ ID NO: 1. More preferably, the polypeptide of the invention has a percentage amino acid identity of greater than about 52%, even more preferably greater than about 70%, and most preferably greater than about 85% compared to the amino acid sequence of SEQ ID NO: 1.

Serpins, such as human  $\alpha_1$ -PI, are single chain proteins containing a  
15 conserved domain structure of about 370 to about 390 residues, usually flanked by amino- or carboxyl- terminal extensions. Those present in plasma are also variably glycosylated, although the carbohydrate side chains are not required for activity. Inhibitory serpins interact with their target proteinase at a reactive site located within a loop structure 30 to 40 amino acids from the carboxyl terminus.  
20 This area is exposed on the surface of the protein and is susceptible to proteolysis by nontarget proteinases. See, for example, Potempa et al., *J. Biol. Chem.*, 269:15957-15960 (1994).

The primary function of members of the serpin family is to neutralize overexpressed serine proteinase activity. This involves highly specific binding  
25 with a target enzyme by complex formation at a pseudo-reactive site peptide bond or "bait region" of the inhibitor. For example, the bait region in human  $\alpha_1$ -PI is the peptide bond between methionine (amino acid residue 358 of SEQ ID NO: 3) and serine (amino acid residue 359 of SEQ ID NO: 3). In the case of human  $\alpha_1$ -PI, the fidelity of this interaction is primarily dictated by the structure  
30 of the methionine residue which is usually in agreement with the cleavage specificity of the proteinase being inactivated. It has been shown that oxidation

of human  $\alpha_1$ -PI converts the methionine amino acid residue to "Met-SO" (Travis et al., Annu. Rev. Biochem., 52:655-709 (1983)). Concomitant with this reaction is a dramatic loss of functional activity. It is now believed that, *in vivo*, this may be a mechanism to regulate elastase inhibitory activity at inflammatory sites. It is believed that the presence of periodontain *in vivo*, could result in elevated levels of HNE clinically detected in periodontal disease and may be a potential virulence factor for *P. gingivalis*. Although it was recently demonstrated that *P. gingivalis* produces a proteinase that rapidly inactivates  $\alpha_1$ -PI, the proteinase was not isolated (Nelson et al., Anal. Biochem., 260:230-236 (1998)) until now.

The present invention provides an isolated polypeptide, preferably an isolated proteinase, and more preferably a cysteine proteinase, that has amidolytic activity as evidenced by the hydrolysis of a peptide bond present in a target polypeptide. In general, the amidolytic activity of the polypeptide can be measured by assay of the cleavage of a target polypeptide in the presence of the polypeptide and a buffer (see, for example, Example 1). Preferably, the polypeptide is isolated from an oral bacteria. "Oral," as used herein, refers to biological material, such as bacteria, that can be found and isolated from the mouth of an animal.

In its nascent form, the isolated polypeptide of the invention contains about 843 amino acid residues and has a molecular weight of about 93 kD (Figure 1). "Nascent," as used herein, refers to a full-length unprocessed 93 kD polypeptide containing amino acid 1 to amino acid 843 of SEQ ID NO: 1 and as shown in Figure 1. The 93 kD polypeptide is unprocessed in that it contains additional amino acids not present in the mature polypeptide of the invention.

As shown in Figure 2, proteolytic processing of the nascent 93 kD polypeptide at amino acid 147 (Arg) and amino acid 629 (Lys) yields a prepro-polypeptide (amino acids 1-147), a heavy chain polypeptide containing a catalytic region (amino acids 148-629), and a light chain polypeptide containing the C-terminal portion of the polypeptide corresponding to amino acid 630 to amino acid 843. As used herein, the "catalytic region" or "catalytic subunit" of the heavy chain polypeptide of periodontain (or any polypeptide described



herein) is a region that is able to cleave, i.e., hydrolyze, a target polypeptide, particularly a nondenatured serpin. For example, in the RSL region of non-denatured human serpin  $\alpha_1$ -PI, periodontain will specifically cleave the peptide bond between glutamic acid and alanine residues (amino acids 354 and 355, respectively, of SEQ ID NO: 3). Typically, cleavage of this peptide bond is observed *in vitro* in a solution containing a Tris buffer in a concentration of about 1 millimolar (mM) to about 500 mM, about a 500 micromolar ( $\mu$ M) to about 100 mM cysteine at a pH of about 7 to about 8 and a temperature of about 18° C to about 42° C. Preferably the solution contains about 50 mM Tris, about 20 mM cysteine maintained at a pH of about 7.4. Although cleavage of the peptide bond may occur at ambient temperature (typically about 25° C to about 30° C), preferably a temperature of about 37° C is employed. Periodontain will also specifically cleave the peptide bond between phenylalanine and leucine residues (amino acids 352 and 353, respectively of SEQ ID NO: 3) under the solution conditions set forth above. Cleavage of this latter peptide bond occurs at about half the catalytic rate as cleavage of the peptide bond between the glutamic acid and alanine residues described above.

Without being bound by any specific theory, periodontain and other polypeptides of the invention are additionally characterized by the nonspecific proteolysis of a target polypeptide in that a specific amino acid residue residing in the polypeptide does not necessarily dictate the specificity of the proteinase. For example, periodontain will cleave any target polypeptide nonspecifically in a denaturing environment. A "denaturing environment," as used herein, includes *in vitro* environment conditions, for example, heat, strong reducing agents, such as dithiothreitol and  $\beta$ -mercaptoethanol, sodium dodecyl sulfate, or other chemical manipulation including carboxymethylate-maleylate treatment. A "denaturing environment" also includes *in vivo* denaturing environment conditions, such *in vivo* denaturing environment conditions may be provided by the catalytic action of other *in vivo* polypeptides, for example, gingipain A and gingipain B.

A "mature polypeptide" of the invention is a heterodimer and contains the heavy polypeptide chain, the light polypeptide chain and has proteolytic

enzymatic activity. The polypeptide is mature in that it lacks the prepro region of the nascent polypeptide, e.g., amino acid 1 to amino acid 147 or SEQ ID NO: 1, and is not associated with a tRNA. The mature polypeptide has a predicted molecular weight of about 70 kD to about 80 kD, and preferably a molecular weight of about 75 kD to about 77 kD as determined by gel filtration. The heavy polypeptide chain typically has a molecular weight of about 50 kD to about 57 kD, and preferably a molecular weight of about 52 kD to about 55 kD as determined by gel filtration. The heavy chain preferably contains the catalytic site of the polypeptide. The mature polypeptide also contains the C-terminal light chain having a molecular weight of about 20 kD to about 25 kD, preferably a molecular weight of about 22 kD to about 24 kD. The C-terminal light chain appears to be devoid of enzymatic activity. Additionally, the mature polypeptide has an isoelectric focusing point (pI) of about 5.1 to about 5.5.

The *in vivo* activity of periodontain may have important physiological significance, as it may lead to the inactivation of many biologically active polypeptides and/or transformation of the activity of other biologically active polypeptides. In addition, hydrolysis of peptide bonds by periodontain in conjunction with general catabolic pathways should allow the complete re-utilization of amino acids by living organisms, including bacteria. However, proteinases from bacterial pathogens, if released into the host environment, may interfere with the physiological functions of biologically active polypeptides and, therefore, contribute to the pathogenicity of infectious disease.

The external (i.e., cell surface) localization and uncontrolled activity of a bacterial proteinase, including periodontain, likely contributes significantly to run-away inflammation in the animal host and the pathological degradation of connective tissue during periodontitis. Periodontain, although unable to degrade proteins such as azocasein, casein, lysozyme, collagen, fibrin, plasminogen, and fibrinogen in nature, may be capable of degrading these proteins *in vitro* if present in a denaturing environment as described above. For example, when lysozyme is denatured, i.e., carboxymethylated-maleylated lysozyme, complete digestion of the reduced form of lysozyme by periodontain rapidly occurs. This characteristic indicates that periodontain will readily cleave denatured or easily

accessible polypeptides, but is typically unable to cleave some polypeptides having defined secondary or tertiary structure, such as the native proteins indicated above; human  $\alpha_1$ -PI being the exception.

Based on the requirement that a denaturing environment is typically  
5 needed for periodontain to actively cleave a target polypeptide, periodontain has been classified as a cysteine proteinase. This observation is further confirmed by the fact that periodontain is readily inhibited by common cysteine proteinase inhibitors selected from the group consisting of dichloroisocoumarin, diisopropylfluorophosphate, leupeptin, tosyl-L-lysine chloromethyl ketone, Phe-  
10 Pro-Arg chloromethyl ketone, Z-Phe-Lys benzoyloxy methyl ketone, idoacetamide and L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64). The ability of E-64 to inhibit periodontain further suggests that periodontain is more closely related to members of the papain family of enzymes than other cysteine proteinases of *P. gingivalis*.

15 The polypeptide of the present invention, preferably a cysteine proteinase, can be used as a source of inhibitors (in addition to those described above), such as inhibitory polypeptides and antibodies, for inhibiting the peptidase activity and thereby possibly reducing periodontitis, loss of tooth attachment and periodontal pocket formation. Antibodies to the cysteine  
20 proteinase of the invention can also be used to identify and/or isolate additional cysteine proteinases. Knowledge of specific cysteine proteinases can also be used to make inhibitory polypeptides against other cysteine proteinases. Such inhibitory polypeptides typically include from about 2 to about 20 amino acids from a polypeptide that the cysteine proteinase has at least some specificity for,  
25 such as the specificity that periodontain has for human  $\alpha_1$ -PI, further associated with a chemical group that inactivates the active site of the proteinase, such as chloromethyl ketone and organo-phosphinate compounds. These inhibitory polypeptides and antibodies may be employed to possibly reduce gingivitis, periodontitis, loss of tooth attachment, and/or periodontal pocket formation.

30 As described herein, an example of a specific cysteine proteinase is the cysteine proteinase periodontain isolated from *P. gingivalis* (SEQ ID NO: 1). Periodontain is a unique proteolytic enzyme, and the isolation and

characterization of this novel bacterial proteinase will facilitate the development of therapies that function to inhibit the activity of this and other bacterial proteinases. Although not intending to be limiting, it is conceivable that periodontain is membrane anchored through a putative signal sequence which is not cleaved but remains as a membrane spanning domain similar to other members of the papain family of cysteine proteinases. Periodontain is most frequently associated with the membrane and outer membrane vesicles in strains 2561 (ATCC Accession No: 33277, Bethesda, MD), W50 (ATCC Accession No: 53978, Bethesda, MD), W12, and 381, despite the fact that it is soluble in strain HG66 (Figure 3). This is also in agreement with the distribution of other *P. gingivalis* proteinases.

A preferred cysteine proteinase of the invention, is the mature polypeptide containing amino acid 148 to amino acid 843 of SEQ ID NO: 1. As shown in Figure 1, it is thought that the catalytic domain of periodontain corresponds to the cysteine and histidine residues residing in the heavy chain of the mature polypeptide.

The invention therefore includes a polypeptide having similarity with the amino acid sequence of SEQ ID NO: 1. The two amino acid sequences (i.e., the amino acid sequence of the polypeptide and the sequence of SEQ ID NO: 1) are aligned such that the residues are aligned to maximize the number of amino acids that they have in common along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to maximize the number of shared amino acids, although the amino acids in each sequence must nonetheless remain in their proper order. The percentage amino acid identity is the higher of the following two numbers: (a) the number of amino acids that the two sequences have in common within the alignment, divided by the number of amino acids in SEQ ID NO: 1, multiplied by 100; or (b) the number of amino acids that the two sequences have in common within the alignment, divided by the number of amino acids in the candidate polypeptide, multiplied by 100.

A polypeptide of the invention also can include an active analog or active fragment thereof of a polypeptide containing amino acid 1 to amino acid 843 of

SEQ ID NO: 1. An active analog or active fragment preferably is characterized by the same amidolytic activity properties with respect to a target polypeptide as is the polypeptide shown in SEQ ID NO: 1.

5 An active analog of the invention includes a polypeptide having one or more amino acid substitutions that do not eliminate amidolytic activity for a target polypeptide. For example, an active analog of the invention is characterized by the ability to cleave any denatured polypeptide and nondenatured serpin polypeptide in the RSL region of the serpin polypeptide.

10 Substitutes for an amino acid in the polypeptides of the invention may be selected from other members of the class to which the amino acid belongs. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and tyrosine. Polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include 15 arginine, lysine, and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Examples of preferred conservative substitutions include Lys for Arg and *vice versa* to maintain a positive charge; Glu for Asp and *vice versa* to maintain a negative charge; Ser for Thr so that a free -OH is maintained; and Gln for Asn to maintain a free NH<sub>2</sub>.

20 Analogs, as used herein, also include modifications. Modified cysteine proteinases include a cysteine proteinase that is chemically and enzymatically derivatized at one or more constituent amino acid, including side chain modifications, backbone modifications, and N and C- terminal modifications including acetylation, hydroxylation, methylation, amidation, and the attachment 25 of carbohydrate or lipid moieties, cofactors, and the like. Modified cysteine proteinases of the invention will retain amidolytic activity with regards to a target polypeptide. For example, a modified cysteine proteinase of the invention will hydrolyze the peptide bond in the RSL region of a human  $\alpha_1$ -PI polypeptide at glutamic acid and alanine, and the phenylalanine and leucine 30 peptide bond as described above and as shown in SEQ ID NO: 1.

Fragments of a cysteine proteinase of the invention include a portion of a cysteine proteinase containing deletions or additions of one or more contiguous

or noncontiguous amino acids such that the resulting polypeptide still retains amidolytic activity with respect to a target polypeptide. An example of an active fragment of a polypeptide of the invention is the catalytic domain of the heavy chain polypeptide containing amino acid 148 to amino acid 629 SEQ ID NO: 1.

5           The polypeptide of the invention can be obtained by several methods. Isolation of the polypeptide present on the surface of a cell producing the proteinase typically requires lysis of the cell followed by purification. Such purification methods are well known in the art and the following represent nonlimiting examples of suitable protein purification procedures: fractionation  
10 on immunoaffinity, ion-exchange, hydroxyapatite, Phenyl-Sepharose HP, MonoQ HR 5/5, or MonoP columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on an ion-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75. Preferably, isolation of the polypeptide  
15 from *P. gingivalis* is accomplished using the parameters set forth in Table 1 (acetone precipitation, Sephadex G-150, Mono-Q FPLC, and TSK gel filtration) or a combination of hydroxyapatite, Phenyl-Sepharose HP, MonoQ HR 5/5 and MonoP column chromatography steps as described herein.

          A cysteine proteinase of the invention may also be isolated from  
20 organisms other than *P. gingivalis*. Other organisms can express a cysteine proteinase that is encoded by a coding region having similarity to the mature polypeptide coding region. A "coding region" is a linear form of nucleotides that encodes a polypeptide, usually via mRNA, when placed under the control of appropriate regulatory sequences. The boundaries of a coding region are  
25 generally determined by a translation start codon at its 5' end and a translation stop codon at its 3' end. "Regulatory region" refers to a nucleic acid fragment that regulates expression of a coding region to which a regulatory region is operably linked. Nonlimiting examples of regulatory regions include promoters, transcription initiation sites, translation start sites, translation stop sites, and  
30 terminators. "Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A regulatory element is "operably linked" to a coding region when it is

joined in such a way that expression of the coding region is achieved under conditions compatible with the regulatory region.

Alternatively, other organisms can express a cysteine proteinase from a recombinant coding region encoding the proteinase. The identification of similar coding regions in other organisms can be accomplished as described herein. A cysteine proteinase can be isolated using purification methods that are well known in the art. Alternatively, the proteinase can be chemically synthesized using methods that are well known in the art including, for instance, solid phase synthesis. Examples of, for instance, coding and regulatory regions are described herein.

The expression of periodontain from *P. gingivalis* and subsequent cleavage of a specific substrate results in a free amino acid or a free leaving group, each of which can be assayed using techniques known to those of skill in the art. Other methods can be based on immunogenic properties of periodontain, for instance immunoassays and histochemistry, the detection of mRNA, and PCR related methods, all of which are known to one of skill in the art.

As described in the Examples, the amino acid sequence of the amino-terminal end of a heavy chain polypeptide was used to identify the nucleotide sequence of the periodontain coding region (SEQ ID NO: 2). The nucleotide sequence was present in a publically available database containing the nucleotide sequence of the partially finished *P. gingivalis* W83 genome. The nucleotide sequence of the genome of this bacterium is currently being determined by the Institute for Genomic Research and is available at [www.tigr.org](http://www.tigr.org). However, even though the nucleotides that encode the *P. gingivalis* cysteine proteinase are known, there has been no indication that the nucleotides were in fact transcribed and translated. The data obtained from the database only contained the nucleotide sequence of a genomic clone; there was no disclosure that the nucleotides did or did not contain an open reading frame. Moreover, there is little data known to the art regarding regulatory regions required for either the transcription or the translation of a nucleotide sequence in *P. gingivalis*.

Thus, a person of ordinary skill, having the nucleotide sequence of the genomic clone, would not be able to predict that the open reading frame encoding periodontain was transcribed or translated. Moreover, even if there was a suggestion that the open reading frame was both transcribed and translated, there is no suggestion that the polypeptide encoded by the open reading frame would have the novel activity of periodontain.

Accordingly, the present invention is directed to a nucleic acid fragment encoding a polypeptide, particularly a cysteine proteinase, active analog (including active modification) or active fragment thereof. The nucleic acid fragment can have a nucleotide sequence as shown in SEQ ID NO: 2. Alternatively, nucleic acid fragments of the invention include those whose complement hybridize to SEQ ID NO: 2 under standard hybridization conditions as described herein. During hybridization the entire nucleotide sequence of the complement can hybridize with SEQ ID NO: 2. Preferably, at least about 15 nucleotides of the complement hybridize with SEQ ID NO: 2, more preferably at least about 50 nucleotides, most preferably at least about 100 nucleotides.

Alternatively, the nucleic acid fragment can have a nucleotide sequence encoding a polypeptide having the amino acid sequence or a fragment thereof as shown in SEQ ID NO: 1. An example of the class of nucleotide sequences encoding such a polypeptide is SEQ ID NO: 2. This class of nucleotide sequences is large but finite, and the nucleotide sequence of each member of the class can be readily determined by one skilled in the art by reference to the standard genetic code.

The identification of similar coding regions in other organisms can be accomplished by screening individual wild-type microorganisms for the presence of nucleotide sequences that are similar to the coding region of periodontain, which is shown in SEQ ID NO: 2. Screening methods include, for instance, hybridization of a detectably labeled probe with a nucleic acid fragment.

Standard hybridizing conditions are a modification of the conditions used by Church et al., Proc. Natl. Acad. Sci., (USA) 81:1991 (1984): 0.5 M phosphate buffer, pH 7.2, 7 % SDS, 10 mM EDTA, at 68° C, and three washes, each for 20



minutes in 2x SSC, 0.1 % SDS, at 65° C. Preferably, a primer will hybridize to the nucleotide sequence set forth in SEQ ID NO: 2 under standard hybridizing conditions. Generally the primer does not have to be complementary to all the nucleotides of the nucleic acid fragment as long as there is hybridization under  
5 the above-stated conditions.

“Complement” and “complementary” refer to the ability of two single stranded nucleic acid fragments to base pair with each other, where an adenine on one nucleic acid fragment will base pair to a thymine on a second nucleic acid fragment and a cytosine on one nucleic acid fragment will base pair to a  
10 guanine on a second nucleic acid fragment. Two nucleic acid fragments are complementary to each other when a nucleotide sequence in one nucleic acid fragment can base pair with a nucleotide sequence in a second nucleic acid fragment. For instance, 5'-ATGC and 5'-GCAT are complementary. The term complement and complementary also encompasses two nucleic acid fragments  
15 where one nucleic acid fragment contains at least one nucleotide that will not base pair to at least one nucleotide present on a second nucleic acid fragment. For instance, the third nucleotide of each of the two nucleic acid fragments 5'-ATTGC and 5'-GCTAT will not base pair, but these two nucleic acid fragments are complementary as defined herein. Typically two nucleic acid fragments are  
20 complementary if they hybridize under the standard conditions referred to herein.

Preferred oligonucleotide primers are nucleic acid fragments complementary to a coding region or another nucleotide sequence that encodes an isolated cysteine peptidase. For instance, a primer can contain a consecutive  
25 series of nucleotides complementary to a portion of the nucleotide sequence encoding the polypeptide or a fragment thereof of SEQ ID NO: 1. Preferably, an oligonucleotide primer is about 15 base pairs, more preferably about 17 base pairs in length. Preferred oligonucleotide primers are degenerative primers. Such degenerative primers can be prepared in accordance with any region of  
30 interest as set forth in SEQ ID NO: 2. Methods of detectably labeling an oligonucleotide primer are well known to the art.

The nucleic acid fragment that is identified by the prepared primer is further analyzed to determine if it encodes a polypeptide with amidolytic activity for a target polypeptide as defined herein. Another method for screening individual microorganisms for the presence of nucleotide sequences that are  
5 similar to the coding regions of the present invention is the polymerase chain reaction (PCR).

Individual wild-type microorganisms containing nucleic acid fragments encoding a polypeptide of the invention can also be identified using an antibody. Preferably the antibody is directed to the cysteine proteinase periodontain. The  
10 production of antibodies to a particular polypeptide is known to a person of skill in the art, and is further detailed herein.

The use of hybridization of an oligonucleotide primer to a coding region present in individual wild-type microorganisms can be used as a method to identify the nucleotides of the coding region identical or similar to a coding  
15 region present in SEQ ID NO: 2. Two nucleic acid sequences are "similar" if the two nucleic acid sequences can be aligned so that a percentage of corresponding residues are identical. The two nucleotide sequences (i.e., the nucleotide sequence of the coding region and the nucleotide sequence of the coding region of SEQ ID NO: 2) are aligned such that the nucleotides of the start  
20 and stop codons are in register, then further aligned to maximize the number of nucleotides that they have in common along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to place the nucleotides of the start and stop codons in register and to maximize the number of shared nucleotides, although the nucleotides in each sequence must  
25 nonetheless remain in their proper order. The percentage nucleotide identity is the higher of the following two numbers: (a) the number of nucleotides that the two sequences have in common within the alignment, divided by the number of nucleotides in SEQ ID NO: 2, multiplied by 100; or (b) the number of nucleotides that the two sequences have in common within the alignment,  
30 divided by the number of nucleotides in the candidate coding region, multiplied by 100. Preferably, two nucleic acid sequences have at least about 50%, more preferably at least about 70%, and most preferably at least about 90% identity.

The coding region can then be isolated and ligated into a vector as described below.

As mentioned above, a nucleic acid fragment of the invention can be inserted in a vector. Construction of vectors containing a nucleic acid fragment of the invention employs standard ligation techniques known in the art. See, e.g., Sambrook et al, *Molecular Cloning: A Laboratory Manual.*, Cold Spring Harbor Laboratory Press (1989) or Ausubel, R.M., ed. *Current Protocols in Molecular Biology* (1994). A vector can provide for further cloning (amplification of the nucleic acid fragment), i.e., a cloning vector, or for expression of the polypeptide encoded by the coding region, i.e., an expression vector. The term vector includes, but is not limited to, plasmid vectors, viral vectors, cosmid vectors, or artificial chromosome vectors. Typically, a vector is capable of replication in a bacterial host, for instance *E. coli*. Preferably the vector is a plasmid.

Selection of a vector depends upon a variety of desired characteristics in the resulting construct, such as a selection marker, vector replication rate, and the like. Suitable plasmids for expression in *E. coli*, for example, include pUC(X), pKK223-3, pKK233-2, pTrc99A, and pET-(X) wherein (X) denotes a vector family in which numerous constructs are available. pUC(X) vectors can be obtained from Pharmacia Biotech (Piscataway, NH) or Sigma Chemical Co. (St. Louis, MO). pKK223-3, pKK233-2 and pTrc99A can be obtained from Pharmacia Biotech. pET-(X) vectors can be obtained from Promega (Madison, WI), Stratagene (La Jolla, CA), and Novagen (Madison, WI). To facilitate replication inside a host cell, the vector preferably includes an origin of replication (known as an "ori") or replicon. For example, ColE1 and P15A replicons are commonly used in plasmids that are to be propagated in *E. coli*.

An expression vector optionally includes regulatory regions (e.g., promoters) operably linked to the coding region. The invention is not limited by the use of any particular promoter, and a wide variety are known. Promoters act as regulatory signals that bind RNA polymerase in a cell to initiate transcription of a downstream (3' direction) coding region. The promoter used in the invention can be a constitutive or an inducible promoter. It can be, but need not

be, heterologous with respect to the host cell. Preferred promoters for bacterial transformation include *lac*, *lacUV5*, *tac*, *trc*, T7, SP6 and *ara*.

An expression vector can optionally include a Shine Dalgarno site (e.g., a ribosome binding site), and a start site (e.g., the codon ATG) to initiate

- 5 translation of the transcribed message to produce the enzyme. It can also include a termination sequence to end translation. A termination sequence is typically a codon for which there exists no corresponding aminoacyl-tRNA, thus ending polypeptide synthesis. The nucleic acid fragment used to transform the host cell can optionally further include a transcription termination sequence.
- 10 The *rrnB* terminators, which is a stretch of DNA that contains two terminators, T1 and T2, is an often used terminator that is incorporated into bacterial expression systems (J. Brosius et al., *J. Mol. Biol.*, 148:107-127 (1981)).

- The nucleic acid fragment used to transform the host cell optionally includes one or more marker sequences, which typically encode a polypeptide
- 15 that inactivates or otherwise detects or is detected by a compound in the growth medium. For example, the inclusion of a marker sequence can render the transformed cell resistant to an antibiotic, or it can confer compound-specific metabolism on the transformed cell. Examples of a marker sequence are sequences that confer resistance to kanamycin, ampicillin, chloramphenicol, and
- 20 tetracycline.

- Antibodies can be produced to a polypeptide having the sequence of SEQ ID NO: 1 or a polypeptide having a percentage amino acid identity of SEQ ID NO: 1 as described herein. Alternatively, antibodies can be made to an antigenic analog or antigenic fragment of a polypeptide having the sequence or a fragment
- 25 sequence thereof of SEQ ID NO: 1. An antigenic analog (including antigenic modifications), and antigenic fragment of a polypeptide derived from SEQ ID NO: 1 is one that generates an immune response in an animal (i.e., it does not eliminate peptide antigenicity in an animal). Analogs and fragments used in this context are as defined above with respect to active analogs and active fragments.

- 30 Accordingly, an aspect of the invention is an immunogenic composition comprising an isolated cysteine proteinase, or an antigenic analog or antigenic fragment thereof, preferably the cysteine proteinase periodontain. The cysteine

proteinase of the invention preferably has amidolytic activity to cleave, i.e., hydrolyze, a peptide bond in a target polypeptide.

The immunogenic composition can further include excipients or diluents that are pharmaceutically acceptable as carriers and compatible with the immunogenic composition. The term "pharmaceutically acceptable carrier" refers to a carrier(s) that is "acceptable" in the sense of being compatible with the other ingredients of a composition and not deleterious to the recipient thereof. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the immunogenic composition may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the immune-stimulating composition.

The immunogenic composition can be used in a method for protecting an animal from a disease caused by *P. gingivalis*. This method includes administering the immunogenic composition and eliciting antibodies to a cysteine proteinase, antigenic analog, antigenic fragment, or antigenic modification. The diseases that can be treated in this manner include periodontal diseases, which includes gingivitis and periodontitis. Clinical hallmarks of periodontitis include lose of tooth attachment and periodontal pocket formation.

Alternatively and preferably, periodontal diseases can be treated by the use of inhibitors of a cysteine proteinase. An inhibitor of a cysteine proteinase, preferably periodontain, can be present in a composition that preferably contains a pharmaceutically acceptable carrier. For instance, inhibitors can be applied systemically, subgingivally (e.g., subgingival irrigation) and/or by controlled release delivery directly into the periodontal pocket using methods well known to the art (see, e.g., Kornman, K., *J. Periodontol.*, 64:782-791 (1993)). Preferably, an inhibitor is applied subgingivally or by controlled release delivery.

The cysteine proteinase, active analogs and active fragments thereof can be used in a method of reducing growth of bacteria *in vitro* or *in vivo*. Preferably, the bacteria is a periodontal pathogen, i.e., a bacterial pathogen that

causes periodontal disease, more preferably the bacteria is *P. gingivalis*. The inability of asaccharolytic *P. gingivalis* to utilize free amino acids makes the bacterium entirely dependant on an external peptide supply. The action of the polypeptides of the invention may be required for bacterial growth, and

5 inhibition of the polypeptides of the invention may inhibit the *in vivo* growth of organisms, including *P. gingivalis*.

The present invention also includes a method for identifying and isolating an inhibitor of a polypeptide of the invention. The method includes identifying an agent that inhibits the amidolytic activity of the polypeptide by  
10 incubating the polypeptide with the agent under conditions that promote amidolytic activity of the polypeptide and determining if the amidolytic activity of the polypeptide is reduced relative to the amidolytic activity of the polypeptide in the absence of the agent.

15

### **Examples**

Objects and advantages of this invention are further illustrated by the following examples, but the particular materials and amounts thereof recited in these examples, as well as other conditions and details, should not be construed to unduly limit this invention.

20

### **Example 1**

25 **Isolation and characterization of a proteinase derived from *P. gingivalis***

#### **Materials and Methods**

##### **Reagents**

Diisopropyl fluorophosphate, leupeptin, and 3,4 dichloroisocoumarin  
30 were purchased from Calbiochem, La Jolla, CA. All other reagents, unless otherwise indicated, were obtained from Sigma Chemical Company, St. Louis, MO. All reagents were of at least analytical grade.

### Bacteria Cultivation

The *P. gingivalis* (HG66) strain (Dr. Roland Arnold, University of North Carolina, Chapel Hill, NC) was used for the purification of periodontain. Cells were grown anaerobically in 5 liters (L) of broth containing 150 grams (g) of trypticase soy broth, 25 g yeast extract (both from Difco, Detroit, MI), 25 milligrams (mg) hemin, 2.5 g cysteine, 0.5 g dithiothreitol (DTT), and 5 mg of menadione (all from Sigma), at 37 °C for 24 hours (h) in an atmosphere of 85% N<sub>2</sub>, 10% CO<sub>2</sub>, and 5% H<sub>2</sub>. The seed culture was used to inoculate 100 L of the same broth and the bacterium was then grown in a 130 L fermentor (W.B. Moore, Inc., Easton, PA) at the University of Georgia Fermentation Plant. Cells were grown for 24 hours until late stationary phase of bacterial growth (OD<sub>660</sub> > 2.0). Additional strains, 2561 (ATCC Accession No: 33277), W50 (ATCC Accession No: 53978) (both from ATCC, Bethesda, MD), W12, and 381 (both from Dr. Caroline A. Genco, Boston University Medical School, Boston MA), were grown under the conditions described above in 1 L volumes.

### Cellular Localization

Whole cell culture mixtures were fractionated to determine localization of periodontain. Initially, a low speed centrifugation (6,000 x g, for 20 minutes at 4 °C) was used to pellet the cells, after which the supernatant was subjected to high speed ultracentrifugation (100,000 x g, for 120 minutes at 4 °C) to separate vesicles from the supernatant which contained all soluble proteins. For quantitation of proteolytic activity all samples were brought to an equal volume by the addition of 50 millimolar (mM) Tris, pH 7.4.

### α<sub>1</sub>-PI Inactivation Assays

Detection of the proteolytic activity of periodontain was determined by following periodontain's ability to specifically inactivate α<sub>1</sub>-PI, using α-chymotrypsin as a target proteinase as described by Nelson et al., Anal. Biochem., 260:230-236 (1998). Briefly, native α<sub>1</sub>-PI (0.15 nanomoles (nmols)) was mixed with samples containing a putative inactivating activity in assay

buffer (50 mM Tris, 10 mM cysteine, pH 7.8 and allowed to incubate at time intervals of 0, 30, 60, 90, 120, 240 minutes after which an equimolar amount of  $\alpha$ -chymotrypsin (0.15 nmols) was added to complex any remaining functional  $\alpha_1$ -PI. The chymotrypsin substrate, N-Suc-Ala-Ala-Pro-Phe- *para*-nitroanalide in dimethyl sulfoxide, was added and, after a 4 minute incubation at 25°C, the reaction was stopped by the addition of 50  $\mu$ l glacial acetic acid. Endpoint absorbance at 405 nm was read in a Molecular Devices SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA) and the percentage of  $\alpha_1$ -PI remaining was calculated from controls (no  $\alpha_1$ -PI, 0.15 nM  $\alpha$ -chymotrypsin) versus (0.15 nM  $\alpha_1$ -PI, 0.15 nM  $\alpha$ -chymotrypsin) in the 4 minute reaction with N-Suc-Ala-Ala-Pro-Phe- *para*-nitroanalide.

Inactivation of  $\alpha_1$ -PI by periodontain was further determined by electrophoresis. Briefly,  $\alpha_1$ -PI (2  $\mu$ M) was incubated with periodontain (2 nM) for 0, 15, 30, 45, and 60 minutes before the reaction was stopped by the addition of an SDS sample buffer. A control sample was preincubated with 100  $\mu$ M of L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64) for 5 minutes prior to 60 minutes incubation with  $\alpha_1$ -PI. The samples were electrophoresed on a 12% separating gel. Subsequently, samples were separated on a 16% peptide gel to isolate a 3 kD fragment produced by this cleavage. Referring to SEQ ID NO: 4 and Figures 4 and 6, both N-terminal amino acid sequence analysis and mass spectroscopy of the isolated fragment revealed that the major cleavage site was after the glutamic acid residue and a minor cleavage site was after the phenylalanine relative to the Methionine-Serine bond which forms the "bait" region of the reactive site loop (RSL) and is attacked by HNE at the (\*) position.

#### Proteinase purification

Bacteria were harvested at stationary phase, and the cells were removed by continuous centrifugation (Sharples AS-16P rotor, Alfa Laval Separation Inc., Warminster, PA). The cell-free culture fluid was concentrated to 5 L in a Pellicon system (Millipore, Bedford, MA) using a 30 kD molecular weight (MW) cut-off membrane, and then precipitated with 7.5 L of acetone at -10 °C.



The protein pellet was redissolved in 20 mM Bis-Tris, 150 mM NaCl, 0.02% NaN<sub>3</sub>, pH 6.8 (buffer A), supplemented with 1.5 mM 4,4'-dithiopyridine disulfide, and dialyzed overnight against the same buffer in a 10 kD MW cut-off membrane (Spectra-Por, Spectrum Corp., Laguna Hills, CA) with two additional  
5 changes of buffer A supplemented with 5 mM CaCl<sub>2</sub>. The dialyzed fraction was clarified by centrifugation (40,000 x g, for 30 minutes), concentrated by ultrafiltration (Amicon PM-10 membrane, Millipore, Bedford, MA) and applied in 20 ml fractions, each representing 5 L of starting supernatant, to a Sephadex G-150 column (5 x 105 centimeters (cm)) (Amersham-Pharmacia, Uppsala,  
10 Sweden) equilibrated with buffer A, at a flow rate of 30 milliliter/hour (ml/h). The activity was pooled, dialyzed against 50 mM Tris, pH 7.4 (buffer B), and further purified by ion exchange chromatography on a Mono-Q column (FPLC system, Amersham-Pharmacia, Uppsala, Sweden), with elution in a linear gradient of 0 mM to 500 mM NaCl in buffer B. Activity was concentrated and  
15 final purification obtained by separation on a TSK-GEL G3000SW (TosoHaas, Montgomeryville, PA) column using 50 mM Tris, 200 mM NaCl, pH 7.4.

## 20 Electrophoresis

Enzyme purification and visualization of the heavy and light chains were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% separating gel using the Tris-HCl/Tricine buffer system, according to Schagger et al., *Anal. Biochem.*, 166:368-379 (1987)). A  
25 nondenaturing PAGE (Davis, *Ann. N.Y. Acad. Sci.*, 121:404-427 (1964)) in a 4-20% gradient gel was used to show the native protein as a single band.

## Molecular Mass Determination

The mass of the native enzyme was determined by gel filtration using a  
30 TSK-GEL G3000SW (TosoHaas, Montgomeryville, PA) calibrated with gel filtration standards (Bio-Rad, Richmond, CA). The mass of the separated heavy and light chains from SDS-PAGE were estimated by scanning the gel using the

Eagle Eye II imaging system (Stratagene, La Jolla, CA) and calculating a linear regression of low molecular weight electrophoresis standards (Amersham-Pharmacia, Uppsala, Sweden) as reference. Accurate molecular mass measurements of the digestion products of  $\alpha_1$ -PI after enzymatic inactivation employed the use of matrix assisted laser desorption ionization (MALDI), with mass spectra acquired using a Vestec MALDI Linear Time-of-Flight Mass Spectrometer (Perspective Biosystems, Perkin-Elmer, Foster City, CA) at the Mass Spectroscopy Facility (University of Georgia, Athens, GA) according to the manufacturer's instructions.

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#### Protein Sequence Analysis

For amino-terminal sequence analysis, proteins resolved by electrophoresis were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane according to Matsudaira (Matsudaira, *J. Biol. Chem.*, **262**(21):10035-10038 (1987)). Sequence analysis was performed with an Applied Biosystems 4760A gas-phase sequencer at the Molecular Genetics Instrumentation Facility (University of Georgia, Athens, GA) operated according to the manufacturer's recommendations.

#### 20 Inhibition studies

For inhibition studies, periodontain was used at a concentration which was capable of completely inactivating 0.15 nmols of  $\alpha_1$ -PI in our standardized assay (Nelson et al., *Anal. Biochem.*, **260**:230-236 (1998)) after one hour of incubation. Representatives of the various classes of proteinase inhibitors, at indicated concentrations, were preincubated with enzyme for 5 minutes prior to the addition of  $\alpha_1$ -PI. The cleavage of inhibitor (%) was normalized to a native inhibitor control in order to give the relative percent inactivation for each compound or protein tested against periodontain. The compound, L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64), which stoichiometrically inhibits cysteine proteinases of the papain family, was used to titrate periodontain. This allowed us to quantitate stock solutions of

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periodontain in terms of active enzyme, and make dilutions to desired concentrations for the degradation experiments described below.

Protein and peptide degradation by periodontain

5       The degradation of proteins was followed by using either native lysozyme or reduced-carboxymethylated-maleylated lysozyme (lysozyme-RCM), (30 micromolar ( $\mu\text{M}$ )). Either protein was incubated with periodontain (30 nM) in a final volume of 20 microliter ( $\mu\text{l}$ ) of assay buffer for 0 and 15 minutes, 1 hour and 24 hours. The reaction was stopped by addition of 20  $\mu\text{l}$  of  
10   SDS sample buffer (4% SDS, 20% glycerol, 0.125 M Tris-HCl, pH 6.8), followed by boiling for 5 minutes, after which the entire sample was electrophoresed on a 12% gel and stained in 0.1% Comassie Brilliant Blue to visualize the protein bands.

      To determine the cleavage sites within the reactive site loop (RSL) of  $\alpha_1$ -  
15   PI, inhibitor (20  $\mu\text{g}$ ) was incubated with 1  $\mu\text{g}$  of periodontain for 4 hours, after which the sample was subjected to 16% SDS-PAGE to separate the approximately 3 kD fragment obtained by cleavage within the loop. The fragment was analyzed for both amino terminal sequence and molecular mass using an automated protein sequencer and mass spectroscopy, respectively, as  
20   described above.

      For analysis of the fragments obtained through digestion of the insulin  $\beta$ -chain, periodontain (8 nM) was incubated with this peptide substrate (40  $\mu\text{M}$ ) in assay buffer in a final volume of 90  $\mu\text{l}$  for desired time intervals. After stopping the reaction by the addition of 10  $\mu\text{l}$  of 10 normal (N) HCL, samples were  
25   centrifuged (10,000 x g, 2 minutes) and the entire supernatant (100  $\mu\text{l}$ ) was subjected to reverse-phase HPLC. Sample application to a Beckman Ultrasphere 5  $\mu\text{m}$  ODS column (4.6 x 250 mm) (Beckman-Coulter Instruments, Fullerton, CA) equipped with an Ultrasphere 5  $\mu\text{m}$  ODS guard-column (4.6 x 45 mm) (Beckman-Coulter Instruments, Fullerton, CA) was carried out in 0.1%  
30   TFA in water (solvent A) and separations were performed with a linear gradient

of 0.08% TFA in 80% acetonitrile/water (solvent B) over 40 minutes at a flow rate of 1 ml/minute. The peptide elution was monitored at 215 nm.

#### Gelatin Zymograph

- 5           Zymography analysis on gelatin gels was performed on pure samples of periodontain in the presence of 5 mM cysteine, with or without 100  $\mu$ M of E-64 (samples were incubated with E-64 for about 5 minutes). After the addition of SDS sample buffer, the samples were subjected to electrophoreses at 4 °C on a 10% SDS-PAGE with gelatin (Difco, Detroit MI) (0.1 mg/ml) incorporated into
- 10 the gel. Following electrophoresis, the gel was washed twice with 2.5% weight/volume (w/v) Triton X-100 to remove the SDS and then incubated in activation buffer (50 mM Tris, 20 mM cysteine, pH 7.4) at 37 °C for two hours. The zymograph was developed in 0.1% Amido Black, with clearing zones (negative staining) indicating proteolytic digestion of the incorporated gelatin.
- 15 The gelatin zymograph indicated that the periodontain heavy chain (55 kD) contained the catalytic active site.

#### Cloning of gene fragment encoding N-terminus of periodontain

- Based on the N-terminal sequence of the heavy chain (55 kD) of
- 20 periodontain (SEQ ID NO: 1), a pair of degenerate oligonucleotide primers (5'-ACNGA(G/A)GGNGTNCCNGC-3') (SEQ ID NO: 7) and (5'-NCGCATNGG(G/A)TC(G/A)TT-3') (SEQ ID NO: 8) corresponding to amino acid residues (TEGPA) (SEQ ID NO: 9) and amino acid residues (NDPMR) (SEQ ID NO: 10), respectively, were designed. The DNA fragment coding for
- 25 the N-terminus of periodontain was amplified by PCR using *Pwo* DNA Polymerase (Boehringer-Mannheim/Hoffman LaRoche Ltd., Basel, Switzerland), and 10 nanograms (ng) of W50 *P. gingivalis* DNA (purified by the Purgene kit, Gentra Systems Inc., Plymouth, MN). PCR was carried out with 500 ng of primers for 1 minute at 94 °C, 1 minute at 65 °C, and 20 seconds at 72
- 30 °C. The expected product of 69 basepairs was purified from a 2% agarose gel with the Ultrafree MC Millipore Filter (Millipore, Bedford, MA), phosphorylated at the 5' end with polynucleotide kinase (PNK), and blunt end

ligated into a *SmaI* digested pUC19 vector. The fragment coding for the N-terminus of periodontain was identified by sequence analysis.

#### Identification of the periodontain gene

5 A database containing the unfinished *P. gingivalis* W83 genome (available from the National Center for Biotechnology Information (NCBI), Unfinished Microbial Genomes at [www.ncbi.nlm.gov](http://www.ncbi.nlm.gov) was searched for the presence of nucleotide sequences corresponding to the NH<sub>2</sub>-terminal amino acid sequences of both chains of periodontain using the TBALSTN algorithm  
10 (Altschul et al., Nucleic Acid Res., 25:3389-3402 (1997)). The sequence of the clone: gnl | TIGR | *P. gingivalis*\_112, which contained the translated sequence for both chains of periodontain, was retrieved from The Institute for Genomic Research (TIGR) database (<http://www.tigr.org>). The gene encoding periodontain was identified using the NCBI open reading frame (ORF) finder  
15 program (also found at NCBI) and the amino acid sequence, obtained by conceptual translation of the entire ORF, was further used for homology screening performed with the NCBI BLAST search tool.

## 20 Results

### Enzyme Purification

Previous experience in purifying enzymes from culture supernatants of *P. gingivalis* indicated that ice cold acetone precipitation as an initial step was successful in separating active proteinases from the bulk of peptides and proteins  
25 present in or released into the growth medium. Similarly, G-150 gel filtration as an early step was also utilized (Chen et al., J. Biol. Chem., 267(26):18896-18901 (1992); Pike et al., J. Biol. Chem., 269(1):406-411 (1994)), both to resolve proteins into rough molecular weight fractions and to remove the excess heme and phytochrome that coprecipitate during acetone treatment. Subsequent anion  
30 exchange chromatography (Mono Q) and gel filtration (TSK) allowed periodontain to be purified to near homogeneity. Our yield of 11%

corresponded to over 1 mg of pure protein per each 5 L of starting culture fluid (Table I).

Table I.

Purification of *P. gingivalis* Periodontain

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Fraction Step	Volume	Total Activity	Total Protein	Specific Activity	Purification	Yield
	ml	Units <sup>a</sup>	mg	units/mg	fold	%
Culture Fluid	15,000	11,000	~300,000	.04	1.00	100
Acetone Precipitation	60	8,100	3,500	2.3	58	74
Sephadex G-150	120	6,550	32.3	203	5,070	60
Mono-Q, FPLC	5	1,958	5.40	363	9,065	18
Gel Filtration, TSK	6	1,200	3.26	368	9,203	11

<sup>a</sup>Based on 1 unit = the activity necessary to completely inactivate 0.15 nmol of  $\alpha_1$ -PI in 60 minutes at 37°C using the assay described above.

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### Physical properties

TSK gel filtration of the pure enzyme yielded a single protein peak that eluted with a MW of approximately 75 kD, based on a linear regression data analysis from standards (data not shown). However, a 10% SDS-PAGE containing 1 µg of pure periodontain yielded two distinct bands at 55 kD (heavy chain) and 20 kD (light chain), suggesting periodontain, as a native protein, is a heterodimer (data not shown). Testing a nondenaturing PAGE containing 1 µg pure periodontain and 5 µg of albumin, albumin formed dimers, and to a lesser extent, trimers when subjected to electrophoresis. Conversely, periodontain, under nondenaturing conditions, did not yield any bands indicating a dimer or trimer (data not shown).

Amino terminal sequence analysis of each of these subunits yielded the sequences of TEGVPAEVHALMDNGHFANDPMR (SEQ ID NO: 11) and DEWKKIGSVSVK (SEQ ID NO: 12) for the heavy (55 kD) and light (20 kD) chains, respectively. Analysis of the single protein species isolated from the nondenaturing PAGE, gave two new amino terminal sequences in equimolar quantities, which corresponded to those described above, confirming that periodontain is a heterodimer. It should also be noted that either heating of the sample and/or presence of SDS in the gel buffer was sufficient to separate the heavy and light chains on electrophoresis, suggesting that the native heterodimer was stabilized by ionic interactions rather than a disulfide bridge or covalent bonds. Isoelectric focusing yielded a pI of 5.3 for the native protein (data not shown) while gelatin zymography indicated that the 55 kD heavy chain contained the catalytic active site but the 20 kD light chain was devoid of enzymatic activity.

### Stability

Periodontain activity was detected over a broad pH range of about 6.0 to about 9.0, with the optimum being between a pH of about 7.5 to about 8.0. The enzyme was stable at 37 °C, overnight, and at 4 °C for several weeks, when stored in the absence of cysteine. The presence of reducing agent resulted in a 50% loss of activity at 37 °C overnight, presumably because of autodigestion. Heating to 60 °C caused complete loss of activity. Samples were routinely stored at -80 °C for several months with less than a 10% loss in activity.

#### Activation and inhibition

Periodontain was completely inactive in the absence of reducing agents, whereas full activity was achieved with either free cysteine,  $\beta$ -mercaptoethanol, dithiothreitol (DTT), or dithioerythritol (DTE) at 0.1 mM concentration. Unlike the gingipains, which have higher activity in free cysteine, no single reducing agent was superior in activating periodontain. Furthermore, increasing concentrations of these reagents (up to 10 mM) did not cause any additional stimulation of periodontain activity. Finally, calcium ions ( $\text{Ca}^{2+}$ ) did not have a stabilizing effect and glycyl-glycine did not stimulate activity, indicating additional differences between periodontain and the gingipains (data not shown). Based on its requirement for a reducing environment to become active, periodontain can be classified as a cysteine proteinase, and this is confirmed by the fact that it is readily inhibited by common cysteine proteinase inhibitors (Table II). The ability of E-64 to inhibit periodontain suggests that this enzyme is more closely related to members of the papain family than other cysteine proteinases of *P. gingivalis* which are either not inhibited (Kgp) or only weakly inhibited (Rgp's) by this compound.



**Table II.**  
**Inhibition Profile of Periodontain**

5	Inhibitor	Class	Concentration	Percent
				Inhibition
10	EDTA	Metallo	25 mM	0
	Dichloroisocoumarin	Serine	0.5 mM	2
	Diisopropylfluorophosphate	Serine	0.5 mM	5
	Leupeptin	Serine/Cysteine	50 $\mu$ M	88
15	Tosyl-L-lysine chloromethyl ketone	Serine/Cysteine	5 mM	91
20	Phe-Pro-Arg chloromethyl ketone	Serine/Cysteine	10 $\mu$ M	96
	Z-Phe-Lys benzoyloxy methyl ketone	Cysteine	10 $\mu$ M	99
	Idoacetamide	Cysteine	5 mM	100
25	E-64	Cysteine	100 $\mu$ M	100

#### Enzyme specificity

30 Periodontain was originally identified as a unique proteinase which was able to inactivate  $\alpha_1$ -PI through proteolytic cleavage. From the 3 kD size difference of cleaved versus native  $\alpha_1$ -PI noted on SDS-PAGE, it was speculated

that periodontain caused hydrolysis within the exposed C-terminal reactive site loop (RSL) of this molecule. Sequencing of the peptide generated by incubation of  $\alpha_1$ -PI with periodontain indicated that cleavage took place after the glutamic acid (amino acid residue 354 of SEQ ID NO: 3), and after phenylalanine (amino acid residue 352 of SEQ ID NO: 3) of the human  $\alpha_1$ -PI. However, screening numerous synthetic pNA substrates with either Glu or Phe specificity in the analogous position yielded no detectable cleavage by periodontain. Indeed, even when the synthetic substrate Phe-Leu-Glu-pNA, which mimics amino acid residues 352, 353 and 354 of the RSL of human  $\alpha_1$ -PI (SEQ ID NO: 3) was employed, no hydrolysis was detected, indicating that a specific amino acid residue at the site of hydrolysis does not dictate the specificity of this enzyme.

Further investigated into the activity of periodontain on protein and peptide substrates was performed to further elucidate cleavage specificity. However, the insulin  $\beta$ -chain was hydrolyzed to such an extent that individual cleavages could not be mapped, even at low E:S molar ratios (1:5000). Rather, the HPLC analysis revealed no less than 10 peptides were generated from this thirty amino acid polypeptide within 15 minute and complete digestion occurred in 60 minutes (data not shown). Next, the activity of periodontain on native proteins was examined. However, the enzyme was unable to degrade azocasein, casein, lysozyme, collagen, fibrin, plasminogen, and fibrinogen (data not shown). In contrast, when lysozyme was reduced, carboxymethylated, and maleylated, complete digestion was noted in less than 10 minutes. These results, together with activity detected on the gelatin zymograph, indicate that periodontain cleaves denatured or easily accessible polypeptide chains, but it cannot cleave whole proteins with defined secondary or tertiary structure,  $\alpha_1$ -PI being the exception.

#### Structure of the Periodontain Gene

The region encoding the N-terminal sequence of the 55 kD catalytic subunit of periodontain was amplified by PCR using degenerate primers and *P. gingivalis* W50 DNA. The 69 basepair PCR product was cloned and sequenced.

Using this structural information, the gene encoding periodontain was extracted from the unfinished microbial genome at NCBI and found to encode an 843 amino acid residue protein with a calculated molecular weight of 93,127 Da (Figure 1). The predicted size of the translated protein is approximately 20 kD larger than that found experimentally by both gel filtration and SDS-PAGE. However, examination of the gene product revealed that proteolytic processing of the translated protein at Arg-147 and Lys-629 would yield a prepropeptide, a heavy chain containing the active site, and a light chain derived from the C-terminal part of the proprotein that corresponds exactly to the experimentally determined amino terminal sequences for these subunits (Figure 2).

Thus, the native protein would have a predicted molecular weight of 76,727 Da, composed of a 52,981 Da catalytic heavy chain and a 23,764 Da C-terminal light chain, with a calculated pI of 5.18. This is in agreement with the experimental findings of a native protein of about 70 to about 80 kD by gel filtration, composed of a catalytic subunit of 55 kD and a noncatalytic subunit of 23 kD by SDS-PAGE, with a pI of 5.3. These data suggest a potential role in the processing of the pro-form of periodontain by both gingipains R and gingipain K, all of which are abundantly present in *P. gingivalis*.

## 20 Distribution of periodontain

Using the cloned 69 basepair catalytic amino terminal fragment as a probe, Southern blot analysis on the W50 strain revealed a single hybridizing band with each of the restriction enzyme digests (data not shown). Furthermore, an  $\alpha_1$ -PI inactivating activity was detected in all *P. gingivalis* strains tested. Interestingly, periodontain was most frequently associated with the membrane and outer membrane vesicles in strains 2561, W50, W12, and 381, despite the fact that it was soluble in strain HG66 (Fig. 3). This is also in agreement with the distribution of other *P. gingivalis* proteinases (Chen et al., *J. Biol. Chem.*, 267(26):18896-18901 (1992); Pike et al., *J. Biol. Chem.*, 269(1):406-411 (1994)).

## Discussion

The multiple trypsin-like proteolytic activities have a significant contribution to the virulence of *P. gingivalis*, for both invasion and host defense evasion. In support of this concept, deletion of the two genes (*rgpA* and *rgpB*) encoding various forms of gingipains R, has been shown to attenuate *in vitro* virulence of the knockout strain (Nakayama et al., J. Biol. Chem., 270:(40):23619-23626 (1995)). In addition, the use of antibodies to gingipains R polypeptide chain derived fragments has shown *in vivo* protection against *P. gingivalis* infection in a mouse model system (Genco et al., Infect. Immun., 66(9):4108-4114 (1998)).

Despite the importance of these bacterial proteinases, the abundance of host derived metallo-proteinases and serine proteinases, including active HNE, in the gingival crevicular fluid of individuals with severe periodontitis, is still believed to be the primary factor responsible for much of the extracellular matrix destruction which occurs in this disease (Cox et al., J. Periodont. Res., 24:353-361 (1989)). The concentration of HNE within the neutrophil is near 3.0  $\mu$ M, and it is likely to be as high as 268  $\mu$ M at sites of inflammation (Travis et al., Am. J. Respir. Crit. Care Med., 150:S143-S146 (1994)), such as those that occur during the development of another major connective tissue disease, pulmonary emphysema.

The regulating inhibitor of HNE is  $\alpha_1$ -PI, a plasma protein which forms a complex with this proteinase and is rapidly removed from the circulation and degraded (Mast et al., J. Biol. Chem., 266(24):15810-15816 (1991)). The inhibitor, however, can itself be inactivated by either oxidation at its reactive site or by proteolytic cleavage by nontarget proteinases within the RSL region (Travis et al., Annu. Rev. Biochem., 52:655-709 (1983)), and it is believed that both mechanisms occur during the development of emphysema. Certainly, the high levels of active HNE in the GCF, despite the presence of  $\alpha_1$ -PI, would suggest that parallel mechanisms for inhibitor inactivation may be also occurring in periodontal disease (Travis et al., Annu. Rev. Biochem., 52:655-709 (1983)). In this respect, it has been reported that whole cells or culture supernatants from

*P. gingivalis* are capable of proteolytically inactivating  $\alpha_1$ -PI (Grenier, Microbiology, 142:955-961 (1996); Carlsson et al., Infect. Immun., 43(2):644-648 (1984)), although it is clear that this is not due to any of the gingipain -R or -K forms since the inhibitor contains no basic residues within its RSL (Potempa et al., J. Biol. Chem., 274(34):21648-21657 (1998)). Thus, another proteinase(s) must be involved in this process, and it is likely that periodontain serves this purpose.

Periodontain was purified from *P. gingivalis* culture fluids and was found to be a cysteine proteinase that is apparently produced as a heterodimer. This enzyme is similar to the other characterized cysteine proteinases from *P. gingivalis* in that all are primarily secreted in strain HG66, yet present on membranes and vesicles in all other strains. Each has a signal peptide sequence, a long prepropeptide, a large catalytic domain, approximately 50 kD, and an additional C-terminal extension (about 20 kD to about 40 kD). In contrast, whereas the gingipains have a restrictive specificity, periodontain is characterized by an apparently nonspecific proteolysis of peptides or denatured proteins. Although the ability of this enzyme to hydrolyze  $\alpha_1$ -PI, a native protein, is contrary to our premise of its inability to cleave proteins with defined structure, this can be explained by the fact that the RSL is present in a flexible, extended conformation protruding above the protein core (Elliott et al., J. Mol. Biol., 275:419-425 (1998)) and, as such, may mimic a denatured protein or peptide.

From analysis of the N-terminus of the catalytic subunit and subsequent cloning of this fragment, it was possible to elucidate the entire gene sequence from the partially completed genome sequence for *P. gingivalis* obtained from NCBI. Searching the database of known sequences has revealed that periodontain is homologous to both *prtT*, a putative cysteine proteinase from *P. gingivalis*, and streptopain (EC 3.4.22.10), a secreted cysteine proteinase from *Streptococcus pyogenes* (Table III and Figure 1). The *prtT* gene, which has recently been cloned and sequenced (Otogoto et al., Infect. Immun., 61(1):117-123 (1993); Madden et al., Infect. Immun., 63(1):238-247 (1995)), is believed to encode a putative proteinase of a 96 kD to a 99 kD protein. It is not surprising

that periodontain and *priT* have highly homologous sequences, being from the same organism. Indeed, *rgpA* and *rgpB* are two completely separate genes that code for almost identical proteins with significantly overlapping specificity.

However, until the gene product for *priT* has been purified and characterized,

5 functional similarities between *priT* and periodontain cannot be assessed.

**Table III**  
**Homology of Periodontain with Streptopain and *priT***  
**(Identity/Similarity)**

Domain	Streptopain	<i>priT</i>
Prepropeptide	27/44	35/56
Catalytic Subunit	37/52	52/69
C-terminal Subunit	N/A	24/42
Full Length Protein	N/A	34/52

Periodontain and *priT*, containing 843 and 840 amino acid residues, respectively, are more than twice the size of the streptopain (398 residues) (Figure 1) The function of the light chain of periodontain is presently unknown; however, the C-terminal domain for the putative *priT* gene product has recently been shown to be identical to the putative product for a hemin regulated gene, *hemR* (Karunakaran et al., *J. Bacteriol.*, 179(6):1898-1908 (1997)). Due to the high homology of the catalytic domains of all three genes, we believe that

periodontain most likely should be classified with *prtT* and streptopain as an additional member of the C10 family of cysteine proteinases, as recently outlined in (Barrett, Rawlings, and Woessner, (eds), Handbook of Proteolytic Enzymes, Academic Press, London (1998)).

5           Because *P. gingivalis* is an asacchrolytic organism, it must acquire both carbon and energy predominantly from proteinaceous sources. Recent evidence using radiolabeled substrates has shown that while this organism is very efficient at taking up dipeptides, it is incapable of transporting single amino acids (which may endogenously be present in GCF) across bacterial cell membranes (Dashper  
10 et al., J. Dent. Res., 77(5):1133 (1998)). This is supported by the fact that despite a large body of research performed on extracellular endoproteinase from *P. gingivalis*, no aminoproteinase or carboxypoteinase have thus far been described.

          It is thought that the gingipains, which only have a specificity for either  
15 Lys-X or Arg-X bonds, would be restricted in their ability to degrade large proteins to the size of dipeptides or tripeptides which could then be transported into the bacterium. Therefore, it is possible that a number of broadly specific proteinases and proteinase, including periodontain, may be physiologically important to *P. gingivalis*, not necessarily as virulence factors, but rather for  
20 nutrient acquisition. This may be accomplished by three pathways. First, since periodontain is the only peptidase in *P. gingivalis* so far described which possesses the ability to inactivate  $\alpha_1$ -PI, this maybe a mechanism for increasing the levels of HNE, a nonspecific host proteinase which might be utilized in protein degradation. Second, in combination with HNE, periodontain may  
25 augment the degradation of peptides produced by the actions of the gingipains. Third, there is evidence that *P. gingivalis* produces a prolyl dipeptidyl peptidase (Kiyama et al., Bioch. Bioph. Acta, 1396:39-46 (1998)), a prolyl tripeptidyl peptidase, and an uncharacterized collagenase. This proteolytic milieu in the GCF could aid in the final production of peptides capable of being taken up by  
30 *P. gingivalis*.

          The complete disclosures of the patents, patent documents, publications, ATCC deposits, and electronically available material (e.g., GenBank amino acid

and nucleotide sequence submissions) etc., cited herein are incorporated by reference in their entirety as if each were individually incorporated. Various modifications and alterations to this invention will become apparent to those skilled in the art without departing from the scope and spirit of this invention. It should be understood that this invention is not intended to be unduly limited by the illustrative embodiments and examples set forth herein and that such examples and embodiments are presented by way of example only with the scope of the invention intended to be limited only by the claims set forth herein as follows.



**WHAT IS CLAIMED IS:**

1. An isolated oral bacterial polypeptide which has amidolytic activity for cleavage of a nondenatured human  $\alpha_1$ -proteinase inhibitor at a reactive site loop region of the inhibitor.
2. The isolated polypeptide of claim 1 wherein the polypeptide has amidolytic activity in a solution comprising about 1 mM to about 500 mM Tris, about 500  $\mu$ M to about 100 mM cysteine maintained at a pH of about 7 to about 8.
3. The isolated polypeptide of claim 1 which is isolated from *Porphyromonas gingivalis*.
4. The isolated polypeptide of claim 1 which is a cysteine proteinase.
5. The isolated polypeptide of claim 1 which has a molecular weight of about 70 kD to about 80 kD as determined by gel filtration.
6. The isolated polypeptide of claim 1 which cleaves the reactive site loop region of the inhibitor represented by SEQ ID NO: 4 between glutamine and alanine.
7. The isolated polypeptide of claim 6 which cleaves the reactive site loop region of the inhibitor represented by SEQ ID NO: 4 between phenylalanine and leucine.
8. An isolated polypeptide which is an oral bacterial cysteine proteinase and has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of the serpin.

9. The isolated polypeptide of claim 8 wherein the polypeptide has amidolytic activity in a solution comprising about 50 mM Tris, about 20 mM cysteine maintained at a pH of about 7.4 at 37°C.
10. The isolated polypeptide of claim 8 which is isolated from *Porphyromonas gingivalis*.
11. An isolated polypeptide which is isolated from *Porphyromonas gingivalis* and has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of the serpin.
12. The isolated polypeptide of claim 11 wherein the polypeptide has amidolytic activity in a solution comprising about 1 mM to about 500 mM Tris, about 500  $\mu$ M to about 100 mM cysteine maintained at a pH of about 7 to about 8.
13. The isolated polypeptide of claim 11 which has a molecular weight of about 70 kD to about 80 kD as determined by gel filtration.
14. The isolated polypeptide of claim 13 comprising an amino acid sequence having a percentage amino acid identity of greater than 37% to that of amino acid 148 to amino acid 843 of SEQ ID NO: 1.
15. The isolated polypeptide of claim 14 comprising an amino acid sequence having a percentage amino acid identity of greater than 52% to that of amino acid 148 to amino acid 629 of SEQ ID NO: 1.
16. The isolated polypeptide of claim 11 which cleaves a target polypeptide nonspecifically.
17. An isolated polypeptide comprising an amino acid sequence having a percentage amino acid identity of greater than 37% to that of SEQ ID NO: 1.

18. An isolated polypeptide comprising an amino acid sequence represented by SEQ ID NO: 1, an active analog or an active fragment thereof.

19. An isolated polypeptide comprising an amino acid sequence having a percentage amino acid identity of greater than 37% to that amino acid 148 to amino acid 843 of SEQ ID NO: 1

20. An isolated polypeptide comprising an amino acid sequence represented by amino acid 148 to amino acid 843 of SEQ ID NO: 1, an active analog or an active fragment thereof.

21. An isolated nucleic acid fragment encoding an oral bacterial polypeptide which has amidolytic activity for cleavage of a nondenatured human  $\alpha_1$ -proteinase inhibitor at a reactive site loop region of the inhibitor.

22. An isolated nucleic acid fragment encoding a polypeptide which is an oral bacterial cysteine proteinase and has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of the serpin.

23. An isolated nucleic acid fragment encoding a polypeptide which is isolated from *Porphyromonas gingivalis* and has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of the serpin.

24. The isolated nucleic acid fragment of claim 23 wherein the nucleic acid has a nucleotide sequence comprising SEQ ID NO: 2.

25. The isolated nucleic acid fragment of claim 23 wherein the encoded polypeptide comprises an amino acid sequence having a percentage amino acid identity of greater than 37% to amino acid 148 to amino acid 843 of SEQ ID NO: 1.

26. The isolated nucleic acid fragment of claim 23 wherein a complement of the nucleic acid fragment hybridizes to SEQ ID NO: 2 under hybridization conditions of

0.5 M phosphate buffer, pH 7.2, 7% SDS, 10 mM EDTA, at 68° C, followed by three 20 minute washes in 2x SSC, 0.1% SDS, at 65° C, wherein at least about 15 nucleotides of the complement hybridize.

27. A method for identifying an inhibitor of a polypeptide which has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of a serpin comprising isolating an agent that inhibits the amidolytic activity of the polypeptide by incubating the polypeptide with the agent under conditions that promote amidolytic activity of the polypeptide and determining if the amidolytic activity of the polypeptide is reduced relative to the amidolytic activity of the polypeptide in the absence of the agent.

28. The method of claim 27 wherein the polypeptide is isolated from *Porphyromonas gingivalis*.

29. An immunogenic composition comprising a polypeptide which has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of a serpin that is capable of eliciting antibodies in an animal.

30. A composition comprising an inhibitor to a polypeptide which has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of a serpin isolated from an oral bacterium.

10/030330  
JC10 Rec'd PCT/PTO 19 OCT 2001

PATENT  
Attorney Docket No. 235.00210101

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): James Travis, et al. )  
Serial No.: Unknown )  
Filed: Even date herewith )  
For: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN )

COMMUNICATION RE. SEQUENCE LISTING

**BOX PCT**

Assistant Commissioner for Patents  
Washington, D.C. 20231

A Sequence Listing in computer readable form and a copy of the Sequence Listing in written form are submitted herewith. The Sequence Listing is identical to the Sequence Listing filed in the International Application. Applicants request entry of same into the specification.

In accordance with 37 C.F.R. §1.821(f), the information recorded in computer readable form is identical to the written Sequence Listing. It is further submitted that the Sequence Listing does not include new matter.

Respectfully submitted,  
JAMES TRAVIS, ET AL.  
By their Representatives,  
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Customer Number 26813

October 19, 2001  
Date  
DLP/mi

By: David L. Provence  
David L. Provence  
Reg. No. 43,022  
Direct Dial (612) 305-1005

Date of Deposit: 19 October 2001  
Express Mail label number: EL 888271957 US  
I hereby certify that this paper and/or fee is/are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to "BOX PCT, Assistant Commissioner for Patents, Washington, D. C. 20231."

Gara L. Ladwig  
Name: Gara L. Ladwig

SEQUENCE LISTING

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TOGTOT" DEEDEDOT

Title: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN

Applicant(s): James Travis, et al.

Serial No. Not yet assigned

Filed: Even date herewith

Docket: 235.00210101

Express Mail No.: EL888271957US

Sheet 1 of 7

10/030330

1/7

Fig. 1a

Fig. 1b

Fig. 1

TOPOT" DEEDOOT

SEQ ID NO:

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1 Periodontain	1 -MKKSFLA	LVMLF	GLAMQGHSA	PVTKEKRALSLAR	--LALRQVSLRMGQTA	VSDKLS	TDY																																																		
Streptopain	61 DIKLDKVNLG	---	GELSGSNMYV	NISTG	-GF	VSC	DKRSPEILGYSTSGS	FDANGKE																																																	
prtT	55 VYK	--AAER	---	EE	---	ALFV	NRGEK	GF	LVAADDRFE	VG	YFKG	FDAAR	P																																												
Periodontain	58 VYRQGD	AERGITSQEEGSPAYFYV	ANRGN	GA	LVAADDRIP	TIL	YSP	IG	FDMDS	M	P																																														
Streptopain	116 N	---	---	IA	SFM	SYVEQ	IKEN	KL	DTYAGTAET	KQ	VKSLLDSKGI	NQG																																													
prtT	105 DNLRG	WLKGY	REMLA	MDCKA	EPI	PI	PEAK	PT	RD	P	SS	IAP	ETGEH	ASDPIL	WDQG																																										
Periodontain	118 DNLR	MMQLQIYDQ	EICL	IL	SKA	QLN	HEIL	---	TEG	PAE	VHA	LMDN	NGHF	ANDP	W	NQG																																									
Streptopain	164 NP	NLLTP	V	EKV	KPG	EQSFV	QQA	AA	TGC	VATA	T	AQIM	KYHN	P	NKGL	K	YTY	TLSS	NNP																																						
prtT	165 YP	ENTL	HP	LLP	---	---	SGQ	AYT	GC	VATA	M	QIM	RHYK	WPE	K	SG	YDY	YDD	MTGT																																						
Periodontain	175 YP	MNKE	PLP	---	---	---	NGH	AYT	GC	VATA	A	AQIM	RYS	HS	W	L	Q	G	EGS	EDYHAG	SLVG																																				
Streptopain	224 YFN	HPK	NL	F	AA	I	S	T	R	Q	YN	W	N	N	L	P	T	Y	S	G	R	-	ES	N	V	Q	K	M	A	S	E	L	M	A	D	V	G	I	S	V	D	M	D	Y	G	P	S	S	G	S							
prtT	216 HT	---	---	H	Y	S	G	T	F	G	E	T	Y	N	W	S	K	M	P	G	N	I	S	V	G	-	I	S	P	E	V	K	A	S	T	F	M	R	D	V	S	F	S	V	N	M	Q	F	A	D	F	G	S	G			
Periodontain	226 N	---	---	W	S	G	T	F	G	E	M	Y	D	W	I	N	M	P	G	N	P	D	L	N	I	T	O	S	Q	V	D	A	Y	A	T	L	M	R	D	V	S	A	S	V	S	M	S	F	E	N	G	S	G				
Streptopain	283 AG	SR	V	Q	R	A	L	E	N	F	G	Y	N	Q	S	V	Q	I	N	R	S	D	S	K	Q	D	W	E	A	Q	I	D	K	E	L	S	Q	N	Q	P	V	Y	Q	G	V	G	K	V	G	H	A	F					
prtT	270 T	S	S	V	E	R	A	L	R	E	T	F	Y	K	K	S	L	R	Y	L	H	R	S	L	L	P	G	K	E	W	K	D	M	I	R	K	E	L	A	E	N	R	P	V	Y	Y	A	G	A	D	G	S	M	G	H	A	F
Periodontain	279 T	S	S	V	V	G	A	L	R	N	F	R	Y	K	K	S	L	Q	L	H	V	R	A	L	V	I	S	Q	E	W	K	D	M	I	R	G	E	L	A	S	G	R	P	V	Y	Y	A	G	N	N	Q	S	L	G	H	A	F
Streptopain	343 V	I	D	G	A	D	G	R	N	F	H	V	N	W	G	W	G	V	S	D	G	F	F	R	L	D	A	L	N	P	S	A	L	G	T	G	G	A	G	F	N	G	Y	Q	S	A	V	G	I	K	P	---	---				
prtT	330 V	C	D	G	Y	H	P	D	G	T	F	H	N	W	G	W	G	G	S	N	G	N	F	Y	L	N	L	N	P	G	S	L	G	T	R	A	D	G	G	S	T	D	Q	E	V	G	I	E	P	A	S	N	E				
Periodontain	339 V	C	D	G	Y	A	S	D	G	T	F	H	N	W	G	W	G	V	S	N	G	F	X	L	T	L	L	S	P	S	L	G	I	G	E	C	I	G	F	I	Y	Q	E	T	G	I	E	P	A	K	T	P					

Fig. 1a

10/030330

TDETOT "DEEDDOT"

390 VP-GIVPDDPTITLYGLOHNMSDEA-LD-SVKIKNIYSTYAGDVKLAVRLTLPNGTETTNPA  
 399 AEAGTDALPIALKDEAEYKSESGLVGYSIYNTGEEQSNLDLGYRLNKADEGEVIEVKT  
 448 VPIVVEDIIGESTGNIIPCSQFAEGKNTILYRTDGMADWKEHILMGLVNKITEV  
 459 SSINISWYG-YGEHPESFSLAPNQLSQGINTILYRRRTGTEQMEPVRHAQGGYVNSLKV  
 508 TMPAGDVAYSVD---ARIVKDGSLSHDLKAYSCKLSATVYNPGTTEFRSRVTFALRN  
 518 NTTDPNNVVTVDNNECKLSVPNSFVADLNSYEHSTLVQFNSDSPDEIRPVAFALST  
 565 --TEGRYFLGRHVEHPGDEGEKVSLLITGLKARAGQYMLVCTGDMESLMEDASMIIE  
 578 GATADDVISLGWMAEVPGGSSN-YPVVWAKDVLTLSEGDYTLMYRFSN--NQKDEMKK  
 623 LASEVAEHTSTHSSLLVASNPQIDLLTVHRANPETLPTFTNEGGATFSCKIEVAIK  
 635 TGSVSVKTPTEYTHPLFEVGHNQSTSTYLLDMAHNRVLPDETKNLG-LPENGELVVFRQ  
 683 AFSET-FFQAKEEHMSAQGETKVLSPETANSPLYTNAELFPDGYIYIVREQG-FWDP  
 694 TQSSGSLWAAQETHKQGETFVYKPVVE-----PIPDGSSYRATLHAFVNGQQQ  
 741 DLFGDYVYRRLTDLSSSDIACKDVSTIVLYPNPAHDXVHVAIPTYAGSTRLFDIQ  
 745 LYLKGRNRYTAKANGTAVEAIE--SSEHIRVFPNPAHDVEISAPCIPQETSILFDS  
 801 GRMQLSTKTESADMRDVERLPKGTIYIVVDMVGKIFIR-  
 803 GKVVMKNSLSALHGRDVSRLPNGAYILKVGYTTKINIVH

prtt  
 Periodontain

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Fig. 1b

10/030330

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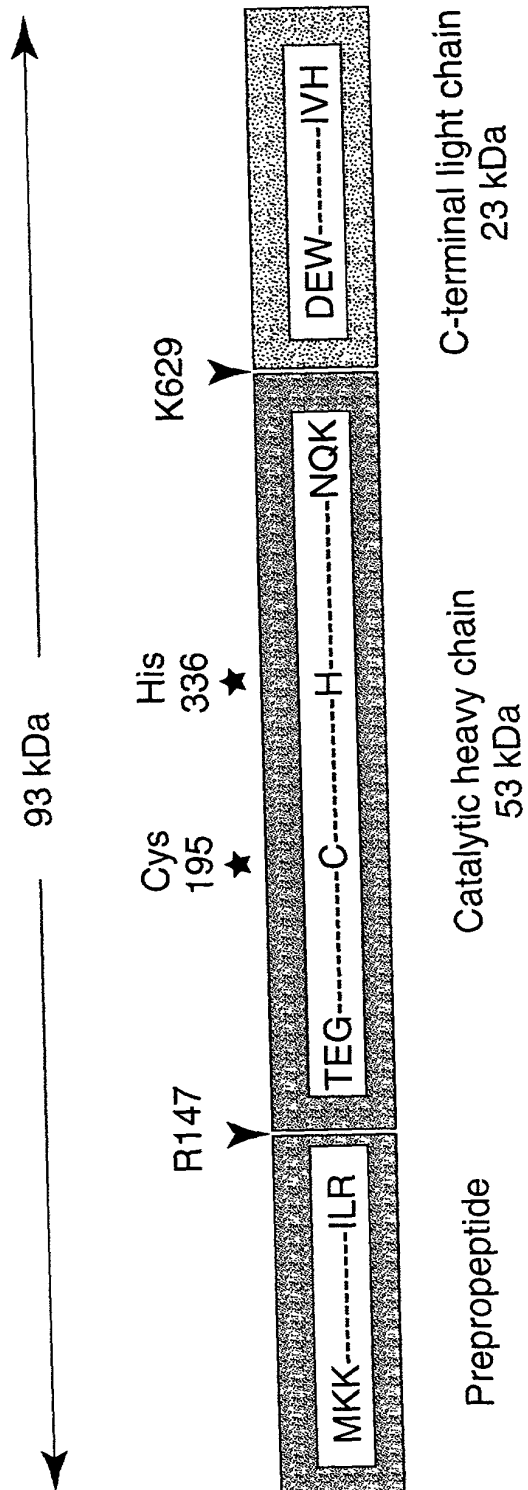


Fig. 2

10/030330

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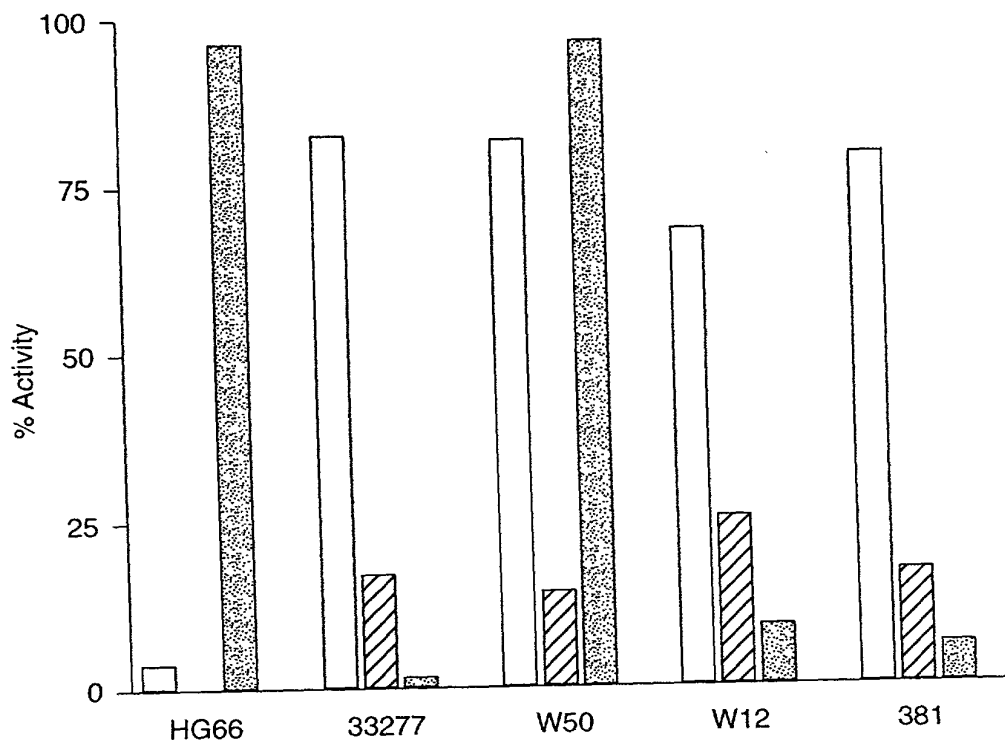


Fig. 3

10/030330

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MPSSVSWGILLAGLCCLVPVSLAEDPQGDAQKTDTSHHQDHP  
TFNKITPNLAFAFSLYRQLAHQSNSTNIFFSPVSIATAFAMLSLGTKADTHDEILEG  
LNFNLTEIPEAQIHEGFQELLRTLNPDSQLQLTTGNGLFLSEGLKLVDFLEDVKKL  
YHSEAFVNFVGDTEEAQKQINDYVEKGTQGKIVDLVKELDRDTVFALVNYIFFKKGWE  
RPFEVKDTEEEEDFHVDQVTTVKVPMMKRLGMFNIQHCKKLSSWVLLMKYLGNATAIFF  
LPDEGKLQHLENELTHDIITKFLENEDRRSASLHLPKLSITGTDLKSVLGQLGITKV  
FSNGADLSGVTEEAPLKLSKAVHKAVLTIDEKGTAAGAMFLEAIPMSIPPEVKFNKP  
FVFLMIEQNTKSPLFMGKVVNPTQK

SEQ ID NO:3

Fig. 4

GAMFLEAIPMSIPPE

SEQ ID NO:4

Fig. 6

106101 DEED001



7/7

[illegible]

SEQ ID NO:2

PATENT  
Docket No. 235.00210101

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): James Travis, et al. )  
Serial No.: Unknown )  
Filed: Even date herewith )  
For: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN )

ELECTION UNDER 37 C.F.R. §3.71, REVOCATION,  
POWER OF ATTORNEY, AND CERTIFICATE UNDER §3.73(b)

Assistant Commissioner for Patents  
BOX PATENT APPLICATION  
Washington, D.C. 20231

Dear Sir:

The Assignee, The University of Georgia Research Foundation, Inc., of the entire interest in the above-identified application hereby elects, under 37 C.F.R. §3.71, to prosecute the application to the exclusion of the inventors.

The Assignee hereby revokes any previous Powers of Attorney and appoints:

Adams, Matthew W.	Reg. No. 43,459
Albin, Loren D.	Reg. No. 37,763
Franklin, Kathleen L.	Reg. No. 47,574
Gebhardt, Mark J.	Reg. No. 35,518
Huebsch, Joseph C.	Reg. No. 42,673
Muetting, Ann M.	Reg. No. 33,977
Provence, David L.	Reg. No. 43,022
Raasch, Kevin W.	Reg. No. 35,651
Sandberg, Victoria A.	Reg. No. 41,287
Walsh, Brian J.	Reg. No. 45,543

as its attorney or agent (with full powers of appointment, substitution and revocation) to prosecute the application, and any division, continuation, continuation-in-part, reexamination or reissue thereof, to make alterations and amendments therein, and to transact all business in the Patent and Trademark Office in connection therewith, and to receive any Letters Patent.

10030330 101001

Applicants: James Travis, et al.

Serial No.: Unknown

Filed: Even date herewith

Title: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN

Please send correspondence to the following address:

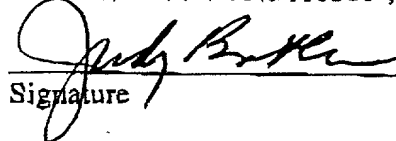
Attention: David L. Provence  
Mueting, Raasch & Gebhardt, P.A.  
P.O. Box 581415  
Minneapolis, MN 55458-1415  
Customer Number 26813

The University of Georgia Research Foundation, Inc., a corporation organized and existing under and by virtue of the laws of the State of Georgia, and having an office and place of business at Boyd Graduate Studies Research Center, Athens, Georgia 30602-7411, USA, certifies that it is the assignee of the entire right, title, and interest in the patent application identified above by virtue of an assignment from the inventors, a copy of which is attached. I have reviewed all the documents in the chain of title of the patent application identified above and, to the best of my knowledge and belief, title is in the assignee identified above.

I am empowered to sign this certificate on behalf of the assignee.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements, and the like so made, are punishable by fine or imprisonment, or both, under Section 1001, Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

THE UNIVERSITY OF GEORGIA  
RESEARCH FOUNDATION, INC.



Signature

Judy C. Butler

Typed or Printed Name

Corporate Secretary

Title

16 October 2001

Date

Declaration

Page 3 of 4

Applicants: James Travis, et al.

Serial No.: Unknown

Filing Date: Even date herewith

Title: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN

Wherefore, we pray that Letters Patent be granted to us for the invention described and claimed in the specification identified above and we hereby subscribe our names to the foregoing specification, claims, and Declaration, on the date indicated below.

1-0  
Name: James Travis Date Feb 28/61  
Citizenship: United States of America GA  
Residence: 825 Riverbend Parkway, Athens, Georgia 30605, USA  
Mailing Address: Same as above  
(If different than Residence)

2-0  
Name: Jan S. Potempa Date  
Citizenship: United States of America GA  
Residence: 170 Barrington Drive, Apt. #102, Athens, Georgia 30605, USA  
Mailing Address: Same as above  
(If different than Residence)

3-0  
Name: Daniel C. Nelson Date  
Citizenship: United States of America NY  
Residence: 504 - East 63rd Street, #20R, New York, New York 10021, USA  
Mailing Address: Same as above  
(If different than Residence)

**ASSIGNMENT**

Whereas We, James Travis, Jan Potempa, and Daniel Nelson, with residences and citizenships as indicated below; have made an invention in

**A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN**

International Application No. PCT/US00/10574, filed April 20, 2000  
U.S. Provisional Appln. Serial No. 60/130,436, filed April 21, 1999

Now, therefore, for good and valuable consideration, receipt of which is acknowledged, we have individually and jointly agreed to assign and transfer and do hereby assign and transfer unto The University of Georgia Research Foundation, Inc. ("Company"), a corporation of GEORGIA, having its principal office at Hoyd Graduate Studies Research Center, Athens, Georgia 30602-7411, USA, its successors and assigns, the entire right, title, and interest in and to said invention and application, and in and to any division or continuation (in whole or in part) of said application, and in and to any and all improvements in said invention made by us or any of us or made jointly with others (provided any such improvement is made during, or within one year after the termination of, the employment by the Company of whichever of us, solely or jointly with one or more others, has made the same), and in and to any and all Patents, reexaminations, reissues, or extensions thereof, of the United States of America and countries foreign thereto (including the right to apply for Patents, Utility Models, or Inventors' Certificates in foreign countries in its own name and to claim any priority rights for such foreign applications to which such applications are entitled under international conventions, treaties, or otherwise), which have been or may be granted thereon or on any continued prosecution application, divisional, continuation (in whole or in part), renewal, reexamination, reissue, or other or further application based in whole or in part upon said invention or improvements thereon, to be held and enjoyed as fully and exclusively as they would have been by us or any of us had this assignment and transfer not been made;

We do further agree for ourselves and for our heirs, executors, and administrators, to execute and deliver without further consideration any further applications, assignments, and documents, and to perform such other acts as we lawfully may, that may be deemed necessary by the Company, its successors, assigns, and nominees, fully to secure its right, title, and interest as aforesaid and to obtain or maintain Patents, Utility Models, or Inventors' Certificates in any and all countries;

Assignment

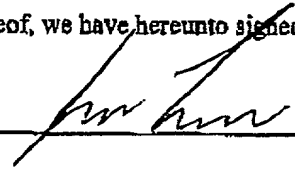
Int'l Appln. No. PCT/US00/10574

Int'l Filing Date: 20 April 2000

For: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN

Page 2 of 3

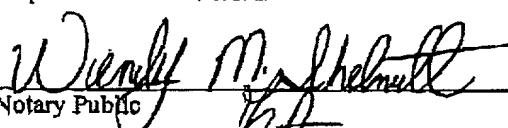
In witness whereof, we have hereunto signed our names on the days and years set forth below.

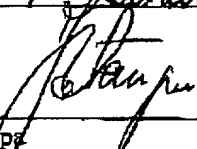
  
Name: James Travis  
Address: 825 Riverbend Parkway, Athens, Georgia 30605  
Citizenship: USA

10/03/00  
Date

STATE OF Georgia )  
 ) ss.  
COUNTY OF Clarke )

On this 3rd day of October 20 00 before me personally appeared James Travis to me known to be the person described in and who executed the foregoing instrument, and he/she executed the same for the uses and purposes therein set forth.

  
Notary Public

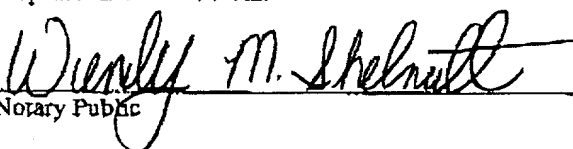
  
Name: Jan Potempa  
Address: 170 Barrington Drive, Apt. #102, Athens, Georgia 30605  
Citizenship: USA

10/03/00

Date

STATE OF Georgia )  
 ) ss.  
COUNTY OF Clarke )

On this 3rd day of October 20 00 before me personally appeared Jan Potempa to me known to be the person described in and who executed the foregoing instrument, and he/she executed the same for the uses and purposes therein set forth.

  
Notary Public

**For: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN**

Daniel Nelson 10/10/00

Name: Daniel Nelson  
Add: 504 E. 4th St. Apt. 205, St. Paul, MN 55101-4205  
Date:

Name: Daniel Nelson  
Address: 504 East 63<sup>rd</sup> Street, Apt. 13-S, New York, New York 10021  
Citizenship: USA

STATE OF New York )  
COUNTY OF New York ) ss.

On this 10<sup>th</sup> day of October 2000 before me personally appeared Daniel Nelson to me known to be the person described in and who executed the foregoing instrument, and he/she executed the same for the usos and purposes therein set forth.

**Notary Public**

✓ **GLORIA CHANG DIGENNARO**  
Notary Public, State of New York  
No. 4884704  
Qualified in Westchester County  
Commission Expires Jan. 12, 2021

**DECLARATION**

We, James Travis, Jan S. Potempa, and Daniel C. Nelson, declare that: (1) our respective citizenships and residence/mailling addresses are indicated below; (2) we have reviewed and understand the contents of the specification identified below, including the claims, as amended by any amendment specifically referred to herein, (3) we believe that we are the original, first, and joint inventors of the subject matter in

**A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN**

Filing Date: Even date herewith

Serial No.: Unknown

described and claimed therein and for which a patent is sought; and (4) we hereby acknowledge our duty to disclose to the United States Patent and Trademark Office all information known to us to be material to the patentability as defined in Title 37, Code of Federal Regulations, §1.56.\*

We hereby claim foreign priority benefits under Title 35, United States Code, §119(e)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate listed below, or §365(a) of any PCT international application which designates at least one country other than the United States of America listed below, and have also identified below any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on the basis of which priority is claimed:

- a. ☒ no such applications have been filed.  
 b. ☐ such applications have been filed as follows:

FOREIGN APPLICATION(S), IF ANY, CLAIMING PRIORITY UNDER 35 USC §119(a)-(d), §365(a), and/or §365(b)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)

ALL FOREIGN APPLICATIONS, IF ANY, FILED BEFORE THE PRIORITY APPLICATION(S)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)

\* Title 37, Code of Federal Regulations, §1.56 is reproduced on the attached page



**Declaration**

**Applicants:** James Travis, et al.

**Serial No.:** Unknown

**Filing Date:** Even date herewith

**Title:** A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN

Page 2 of 4

We hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

- a. ☐ no such applications have been filed.  
b. ☒ such applications have been filed as follows:

PROVISIONAL APPLICATION(S), IF ANY, UNDER 35 USC §119(e)	
APPLICATION NUMBER	DATE OF FILING (day, month, year)
60/130,436	21 April 1999

We hereby claim the benefit under Title 35, United States Code, §120 of any United States applications or §365(c) of any PCT international application(s) designating the United States of America, listed below.

- a. ☐ no such applications have been filed.  
b. ☒ such applications have been filed as follows:

APPLICATION NUMBER	DATE OF FILING (day, month, year)	STATUS (patented, pending, abandoned)
PCT/US00/10574	20 April 2000	Pending

Insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, §112, we acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

The undersigned declare further that all statements made herein of their own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

*Declaration*

*Applicants: James Travis, et al.*

*Serial No.: Unknown*

*Filing Date: Even date herewith*

*Title: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN*

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§ 1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is cancelled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

- (1) Prior art cited in search reports of a foreign patent office in a counterpart application, and
- (2) The closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

- (1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or
- (2) It refutes, or is inconsistent with, a position the applicant takes in:
  - (i) Opposing an argument of unpatentability relied on by the Office, or
  - (ii) Asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

- (1) Each inventor named in the application;
- (2) Each attorney or agent who prepares or prosecutes the application; and
- (3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.

(e) In any continuation-in-part application, the duty under this section includes the duty to disclose to the Office all information known to the person to be material to patentability, as defined in paragraph (b) of this section, which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

**DECLARATION**

We, James Travis, Jan S. Potempa, and Daniel C. Nelson, declare that: (1) our respective citizenships and residence/mailling addresses are indicated below; (2) we have reviewed and understand the contents of the specification identified below, including the claims, as amended by any amendment specifically referred to herein, (3) we believe that we are the original, first, and joint inventors of the subject matter in

**A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN**

Filing Date: Even date herewith

Serial No.: Unknown

described and claimed therein and for which a patent is sought; and (4) we hereby acknowledge our duty to disclose to the United States Patent and Trademark Office all information known to us to be material to the patentability as defined in Title 37, Code of Federal Regulations, §1.56.\*

We hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate listed below, or §365(a) of any PCT international application which designates at least one country other than the United States of America listed below, and have also identified below any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on the basis of which priority is claimed:

- a. ☒ no such applications have been filed.  
 b. ☐ such applications have been filed as follows:

FOREIGN APPLICATION(S), IF ANY, CLAIMING PRIORITY UNDER 35 USC §119(a)-(d), §365(a), and/or §365(b)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)

ALL FOREIGN APPLICATIONS, IF ANY, FILED BEFORE THE PRIORITY APPLICATION(S)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)

\* Title 37, Code of Federal Regulations, §1.56 is reproduced on the attached page.

**Declaration**

Page 2 of 4

Applicants: James Travis, et al.

Serial No.: Unknown

Filing Date: Even date herewith

Title: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN

We hereby claim the benefit under Title 35, United States Code §119(c) of any United States provisional application(s) listed below.

- a. ☐ no such applications have been filed.  
b. ☒ such applications have been filed as follows:

PROVISIONAL APPLICATION(S), IF ANY, UNDER 35 USC §119(e)	
APPLICATION NUMBER	DATE OF FILING (day, month, year)
60/130,436	21 April 1999

We hereby claim the benefit under Title 35, United States Code, §120 of any United States applications or §365(c) of any PCT international application(s) designating the United States of America, listed below.

- a. ☐ no such applications have been filed.  
b. ☒ such applications have been filed as follows:

APPLICATION NUMBER	DATE OF FILING (day, month, year)	STATUS (patented, pending, abandoned)
PCT/US00/10574	20 April 2000	Pending

Insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, §112, we acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

The undersigned declare further that all statements made herein of their own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

**Declaration**

**Applicants:** James Travis, et al.

**Serial No.:** Unknown

**Filing Date:** Even date herewith

**Title:** A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN

Wherefore, we pray that Letters Patent be granted to us for the invention described and claimed in the specification identified above and we hereby subscribe our names to the foregoing specification, claims, and Declaration, on the date indicated below.

Name: James Travis Date  
 Citizenship: United States of America  
 Residence: 825 Riverbend Parkway, Athens, Georgia 30605, USA  
 Mailing Address: Same as above  
 (If different than Residence)

Name: Jan S. Potempa Date  
 Citizenship: United States of America  
 Residence: 170 Barrington Drive, Apt. #102, Athens, Georgia 30605, USA  
 Mailing Address: Same as above  
 (If different than Residence)

Name: Daniel C. Nelson Date  
 Citizenship: United States of America  
 Residence: 504 - East 63rd Street, #20R, New York, New York 10021, USA  
 Mailing Address: Same as above  
 (If different than Residence)

FOOTNOTES

Applicants: James Travis, et al.

Serial No.: Unknown

Filing Date: Even date herewith

Title: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN

## § 1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is cancelled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

- (1) Prior art cited in search reports of a foreign patent office in a counterpart application, and
- (2) The closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

- (1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or
- (2) It refutes, or is inconsistent with, a position the applicant takes in:
  - (i) Opposing an argument of unpatentability relied on by the Office, or
  - (ii) Asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

- (1) Each inventor named in the application;
- (2) Each attorney or agent who prepares or prosecutes the application; and
- (3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.

(e) In any continuation-in-part application, the duty under this section includes the duty to disclose to the Office all information known to the person to be material to patentability, as defined in paragraph (b) of this section, which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

**DECLARATION**

We, James Travis, Jan S. Potempa, and Daniel C. Nelson, declare that: (1) our respective citizenships and residence/mailling addresses are indicated below; (2) we have reviewed and understand the contents of the specification identified below, including the claims, as amended by any amendment specifically referred to herein, (3) we believe that we are the original, first, and joint inventors of the subject matter in

**A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN**

Filing Date: Even date herewith

Serial No.: Unknown

described and claimed therein and for which a patent is sought; and (4) we hereby acknowledge our duty to disclose to the United States Patent and Trademark Office all information known to us to be material to the patentability as defined in Title 37, Code of Federal Regulations, §1.56.

We hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate listed below, or §365(a) of any PCT international application which designates at least one country other than the United States of America listed below, and have also identified below any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on the basis of which priority is claimed:

- a. ☒ no such applications have been filed.  
 b. ☐ such applications have been filed as follows:

FOREIGN APPLICATION(S), IF ANY, CLAIMING PRIORITY UNDER 35 USC §119(a)-(d), §365(a), and/or §365(b)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)

ALL FOREIGN APPLICATIONS, IF ANY, FILED BEFORE THE PRIORITY APPLICATION(S)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)

**Declaration**

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Applicants: James Travis, et al.

Serial No.: Unknown

Filing Date: Even date herewith

Title: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN

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Declaration

Page 3 of 4

Applicants: James Travis, et al.

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(If different than Residence)

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Residence: 170 Barrington Drive, Apt. #102, Athens, Georgia 30605, USA  
Mailing Address: Same as above  
(If different than Residence)

Name: Daniel C. Nelson Date  
Citizenship: United States of America  
Residence: 504 - East 63rd Street, #20R, New York, New York 10021, USA  
Mailing Address: Same as above  
(If different than Residence)

FOOTNOTED

Declaration

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Applicants: James Travis, et al.

Serial No.: Unknown

Filing Date: Even date herewith

Title: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN

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- (1) Prior art cited in search reports of a foreign patent office in a counterpart application, and
- (2) The closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

- (1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or
- (2) It refutes, or is inconsistent with, a position the applicant takes in:
  - (i) Opposing an argument of unpatentability relied on by the Office, or
  - (ii) Asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

- (1) Each inventor named in the application;
- (2) Each attorney or agent who prepares or prosecutes the application; and
- (3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.

(e) In any continuation-in-part application, the duty under this section includes the duty to disclose to the Office all information known to the person to be material to patentability, as defined in paragraph (b) of this section, which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

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